Cellular and molecular mechanisms of glioma growth control

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Abbreviations

Amp	Ampicillin resistance
BBB	Blood brain barrier
Bp	Basepair
BSA	Bovine serum albumin
cDNA	Copy DNA
CMV	Cytomegalovirus
CO_2	Carbondioxide
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
et al.	*et alii* (lat.: and others)
e.g.	*exempli gratia* (lat.: for example)
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence Assisted Cell Sorting
FITC	Fluorescein-5-isothiocyanate
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GSC	Glioma stem cell
HRP	Horseradish peroxidase
Ig	Immunoglobulin
MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
NPC	Neural precursor cell
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
Rpm	Revolutions per minute
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SVZ	Subventricular zone

TfR	Transferrin Receptor
TRITC	Tetramethylrhodamine isothiocyanate
ON	Overnight
UV	Ultraviolet
Wt	Wildtype

Abstract

Part-1: Bone morphogenetic protein-7 release from endogenous neural precursor cells suppresses the tumorigenicity of glioma stem cells.

Gliomas cells with stem-like properties (GSCs) control tumor growth and recurrence. Here, I showed that endogenous neural precursor cells (NPCs) perform an anti-tumor response by specifically targeting GSCs: In vitro, NPCs predominantly expressed BMP7; BMP7 was constitutively released from neurospheres and induced canonical BMP-signaling in GSCs. Exposure of human and murine GSCs to neurosphere-derived BMP7 increased GSC differentiation, attenuated GSC-marker expression, GSC self-renewal and the ability for tumor initiation. NPC-derived BMP or recombinant BMP7 reduced glioma expansion from GSCs. This anti-tumor response of NPCs protect the brain from gliomas by releasing BMP7, which acts as a paracrine tumor suppressor that represses proliferation, self-renewal and tumor-initiation of GSCs.

Part-2: Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells

Transferrin receptors (TfR) are overexpressed in brain tumors, but the pathological relevance has not been fully explored. Here, I showed that TfR is an important downstream effector of ets transcription factors that promotes glioma proliferation and increases glioma-evoked neuronal death. TfR mediates iron accumulation and reactive oxygen formation and thereby enhanced proliferation in clonal human glioma lines, as shown by the following experiments: (1) downregulating TfR expression reduced proliferation in vitro and in vivo; (2) forced TfR expression in low-grade glioma accelerated proliferation to the level of high-grade glioma; (3) iron and oxidant chelators attenuated tumor proliferation in vitro and tumor size in vivo. TfR-induced oxidant accumulation modified cellular signaling by inactivating a protein tyrosine phosphatase (low-molecular-weight protein tyrosine phosphatase), activating mitogen-activated protein kinase and Akt and by inactivating p21/cdkn1a and pRB. Inactivation of these cell cycle regulators facilitated S-phase entry. Besides its effect on proliferation, TfR also boosted glutamate release, which caused *N*-methyl-D-aspartate-receptor-mediated reduction of neuron cell mass. Overall my results indicate that TfR promotes glioma

progression by two mechanisms, an increase in proliferation rate and glutamate production, the latter mechanism providing space for the progressing tumor mass.

Key words:

Glioma stem cells, BMP-7, Transferrin receptor, Ets, Reactive oxygen species.

1. Introduction

1.1. Brain tumors and their classification

Glia cells are the most common cell type in the brain and make up 90 % of the total cell number (Kettenmann, H *et al*, 1995) depending on species. They were discovered by Virchow (1856), who described them as Nervenkitt, a kind of glue for neurons (gr. glia: glue). Initially, they were considered as merely supporting cells for neurons, yet recently they were shown to fulfill a range of far more complex functions. The group of glia cells consists of astrocytes, oligodendrocytes and Schwann cells (Kettenmann, H *et al*, 1995). Historically, brain tumors were thought to consist of transformed glia cells and are therefore called gliomas. Different types of gliomas are astrocytomas, oligodendrogliomas and schwannomas, depending on the preferential type of differentiation of these tumors. Schwannomas often correspond to benign tumors. It is still unknown how these transformations occur and what triggers them. One theory claims that disruptions in the glial cell cycle lead to glioma formation. However, recent research provided more and more evidence that gliomas emerge from neural precursor cells.

Gliomas are the most common group of primary tumors in the brain and make up 30 – 40 % of all brain tumors (Kleihues, P *et al*, 1993). The World Health Organisation (WHO) introduced a classification in 1993, which divides astrocytomas into four malignancy grades:

Tab. 1.1. The World Health Organization (WHO) grading system for astrocytomas

Grade	Example	Criteria
WHO I	Pilocytic astrocytoma Myxopapillary Ependymoma/Subendymoma	Low proliferating, discrete, non invasive tumor
WHO II	Diffuse astrocytoma Papillary, cellular and clear cell Ependymoma	Modest proliferating, partly invasive tumor
WHO III	Anaplastic astrocytoma Anaplastic ependymoma	Fast proliferating, invasive tumor
WHO IV	Glioblastoma multiforme Highly malignant glioma-like Pineoblastoma and Medulloblastoma	Rapidly proliferating, highly invasive tumor

In the present work research and conclusions will be restricted to cells representing glioblastoma multiforme (GBM) or glioma, i.e. a grade IV brain tumor.

1.2. Epidemiology of gliomas

Gliomas or glioblastomas occur with an incidence of 5 in 100,000 (Friese, M.A.*et al*, 2004). The peak of onset of glioblastomas is around 50 - 55 years, which makes them a strongly age-related pathology. Men are slightly more prone to these neoplasms. Furthermore, the incidence is 2 - 3 times higher in white than in black people. Prognosis is poor and the median survival has remained at 9 to 12 months for decades (Stupp, R *et al*, 2005), only few patients survive for three or more years. Main risk factors are high dose radiation, hereditary syndromes and increasing age. Although the last years have revealed some major approaches to develop new surgical and radiation techniques as well as multiple antineoplastic drugs, a cure for glioblastoma remains elusive (DeAngelis, L.M. *et al*, 2001).

1.3. Diagnosis and treatment of gliomas

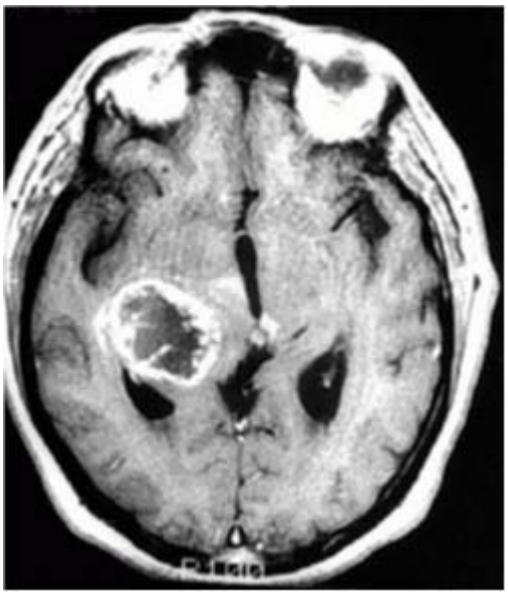


Fig. 1.3. MRT of a human brain, revealing a glioblastoma multiforme
(rad.usuhs.mil/rad/who/zs224248)

If a neurological examination points to a brain tumor, additional testings will be made. These mainly include scans like magnetic resonance imaging (MRI, Fig. 1.3.), computer tomography (CT) or positron emission tomography (PET). In most cases therapy starts with surgical removal of the tumor. Since for the tumor patient gliomas may be located in brain areas which are of vital importance and the surgical removal of these neoplasms can be much more difficult than removing a tumor in other parts of the body. Even if the surgery is successful it has to be assumed that tumor cells have already spread throughout the brain and may be the source for tumor relapses.

One of the main properties of glioma cells is their invasive behaviour, which also signifies the biggest challenge regarding therapy (Holland, E.C. *et al*, 2001; Kleihues, P *et al*, 1995). Therefore combined radiochemotherapy typically follows surgery. Conventional radiation therapy uses X- or gamma-rays but also other types of radiation are available. At present, the standard chemotherapeutic is temozolomide (Temodal®). Its cytotoxicity is due to alcylation of the nucleobase guanine. Although many efforts have been made during the last years to improve

the existing therapies, the biggest problem is still the extreme invasive nature of glioblastomas. It is virtually impossible to prevent migration of tumor cells into the adjacent brain tissue, which may be the primary cause for relapses.

1.4. Pathophysiology of gliomas

Glioma or Glioblastoma multiforme (GBM) consist of a heterogenous mixture of poorly differentiated neoplastic astrocytes (Holland, E.C.*et al*, 2001). They can occur as primary, which means *de novo* tumors but can also, although less frequent, develop from lower grade astrocytomas and thus are defined as secondary tumors. The latter typically develop in younger patients (< 45 years) whereas *de novo* tumors arise almost solely in elderly patients (around 65 years). The tumor as such forms a solid mass from which neoplastic cells are disseminating into the adjacent brain tissue. The tumor itself can reach a considerable size and squeeze out larger amounts of brain mass (Fig. 1.4.), which usually leads to diverse neurological defects.

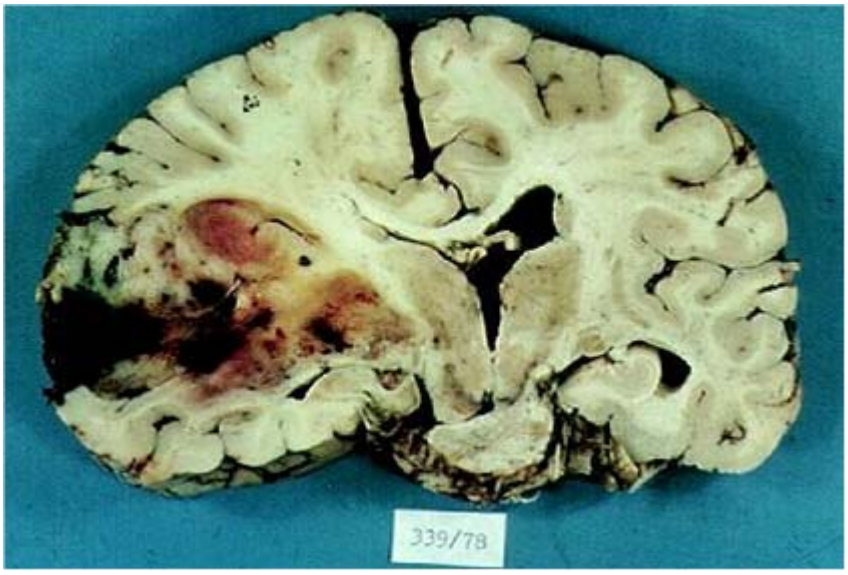


Fig. 1.4. Macroscopic view of glioblastoma multiforme in a human brain (www.neuropat.dote.hu/jpeg/tumor/3gliobl1).

Although primary and secondary tumors differ on the genetic level in many ways, there are some common genetic abnormalities, which are considered as hallmarks of glioblastomas. One of them is the loss of heterozygosity (LOH) on chromosome 10, which seems to be specific for grade IV brain tumors. Very well known are

mutations in the tumor suppressor gene p53 on chromosome 9, which also plays a pivotal role in other types of cancer. In fact, only about a third of glioblastomas carries this mutation, which corresponds to the percentage in lower grade gliomas. However, recent reports indicated that p53 mutations may be much more common, and these mutations can be very complex and may have been overlooked (Zheng, H *et al*, 2008). This suggests that the p53 gene is involved rather early in neoplastic transformation (Kleihues, P., *et al***,** 1993). In about one third of all GBMs one can find amplification and mutation of the endothelial growth factor receptor gene (EGFR) like EGFRvIII which is the mutant version of EGFR in Glioblastoma multiforme mutation which leads to increased cell proliferation. Furthermore platelet-derived growth factor alpha (PDGF-α) and phosphatase and tensin homolog deleted on chromosome ten (PTEN) are two more genes, of which the expression is altered in GBMs (Knobbe,C.B**.** *et al***,** 2002; Nakamura, J.L. *et al*, 2007). PDGF-α belongs to the family of growth factors and is involved in the regulation of cell growth and cell division. It plays a particular role in angiogenesis, which is characteristically increased in cancer to provide sufficient nutrition supply for the tumor. The phosphatase PTEN is a tumor suppressor, which is related to a variety of biological functions like apoptosis, inflammation and immunity. These genetic defects have an effect on other cell proteins and finally result in tumor formation.

1.5. Cell of origin of gliomas

The identification of the cellular origin of gliomas presents an opportunity for improving our understanding of this disease. Recent work in both animal and primary gliomas suggests the more likely possibility that malignant gliomas arise from neural proginator cells. Overexpressing oncogenic Ras and Akt in progenitor cells results in mouse brain tumors that are histologically similar to their human counterparts, but targeting more mature astrocytic progenitors is not equally oncogenic (Holland, E.C. *et al* 2000). Targeting platelet-derived growth factor (PDGF)-B expression to progenitor cells results in tumors identical to human oligodendrogliomas (Dai, C *et al* 2001). In addition, oncogenic events such as *CDNK2A/p16* deletion can drive more mature astrocytic cells to cells that have a progenitor phenotype, with multipotent differentiation potential (Bachoo, R.M. *et al* 2002). Furthermore, malignant gliomas likely contain tumor stem cells, a

relatively primitive population responsible for populating and repopulating the tumors as they develop and progress (Galli, R., *et al* 2004, Singh, S.K., *et al*, 2004 and 2003,); such tumor stem cells may be transformed variants of normal neural progenitor cells. The existence of these tumor stem cells may have major therapeutic implications as well because therapies that do not ablate the tumor stem cells will ultimately prove ineffective in eradicating the tumor.

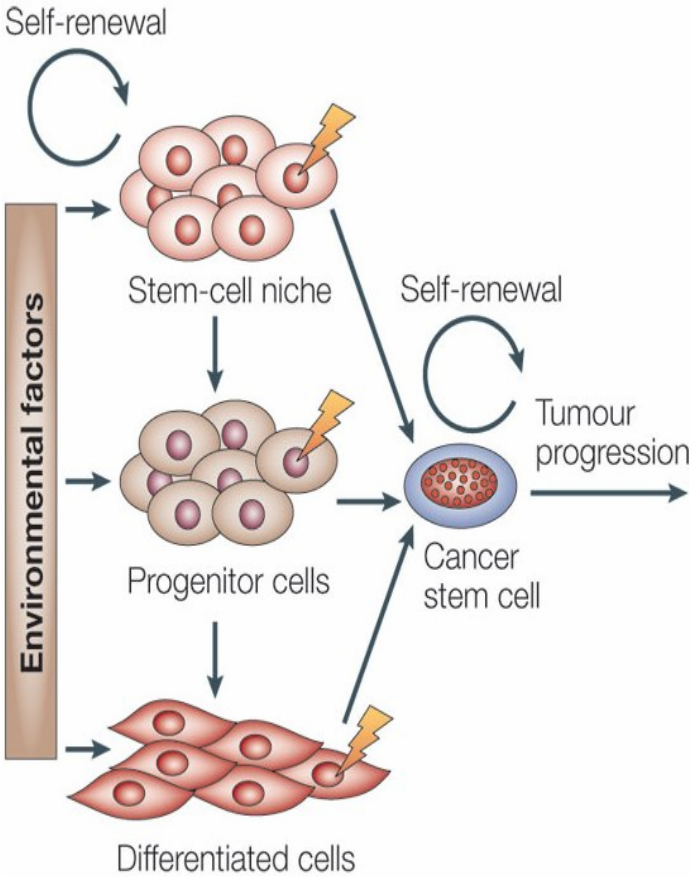


Fig. 1.5: Mutations in stem cells and/or progenitor cells might give rise to glioma stem cells. The cancer stem cell might appear after mutations in specific stem cells or early stem cell progenitors. It is also possible that cancer stem cells can be derived from differentiated cells. There might be numerous factors in the host microenvironment that trigger the initial steps of tumor formation. (Modified from Bjerkvig R, *et al*, 2005).

1.6. Glioma Stem cells (GSCs)

In the past few years, stem cell-like tumor cells have been identified in gliomas (Singh, S.K *et al* 2004). They have been consecutively termed glioma stem cells, brain tumor stem cells or brain tumor initiating cells and identification of these cells has created a paradigm shift in brain tumor research. They are characterized by unlimited capacity for self-renewal, high proliferation, increased ability for

tumor initiation, multi-differentiation and expression of stem cell markers such as CD133 (prominin-1) and nestin. CD133 is a cell membrane glycoprotein and nestin is an intermediate filament protein that is produced in stem/progenitor cells during development and is a marker of proliferating and migrating cells. CD133 and nestin are currently the most accredited markers for the identification of neural stem cells. Their use in glioma stem cell research has been fundamental to reveal the biological properties of glioma stem cells, such as tumor progression and resistance to ionizing radiation or chemotherapy. As a new concept for brain tumor therapy it may be useful to target these CD133 positive glioma stem cells.

1.7. Glioma stem cells may represent as novel therapeutic targets

Current chemotherapeutic strategies involve using non-specific cytotoxic agents that target rapidly cycling cells. Although this may reduce disease burden in many cases, these therapies may miss the rare, self-renewing population that truly gives rise to the malignancy (GSCs) and which is usually non-responsive to standard chemotherapy (Lu C *et al*, 2008) Dissecting the molecular mechanisms that underlie the ability of these cells to self-renew and maintain quiescence may allow the development of novel therapeutic strategies that will allow for more efficacious and less toxic therapies for these devastating tumors.

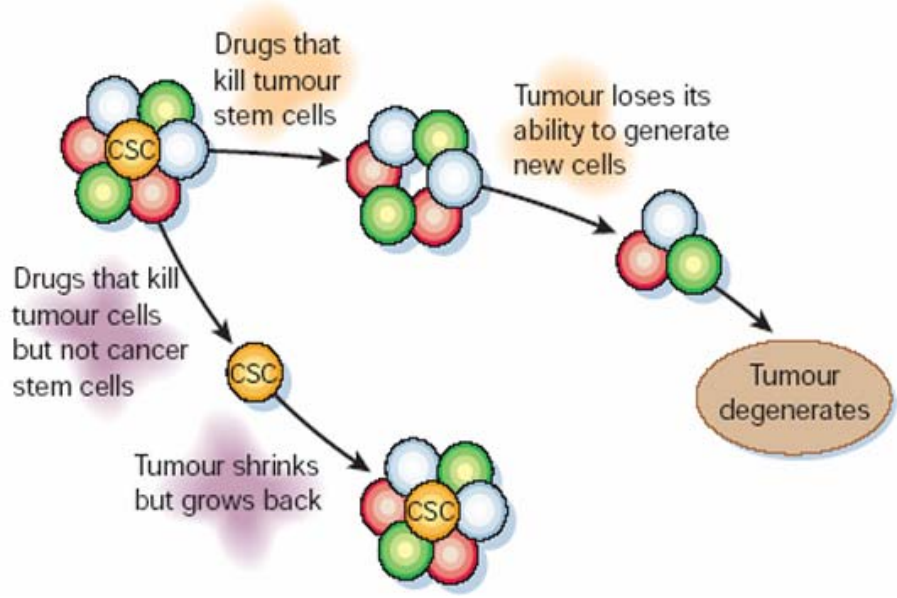


Fig.1.7: Glioma stem cells may represent as novel therapeutic targets: Modified from (Reya et 2001). Conventional therapies shrink tumors by killing mainly cells with limited proliferative

potential, but cancer stem cells may re-establish the tumor. By contrast, therapies targeting cancer stem cells render the tumors unable to maintain themselves or grow (CSC- Cancer stem cell).

CD133 positive human glioblastoma cells were shown to be resistant to radiation therapy, retaining a clonogenic and tumorigenic potential. CD133 positive cells increase in number after irradiation of glioblastoma cells in culture and in tumors growing in vivo. The CD133 positive cells undergo similar DNA damage to those of their CD133 negative counterparts, but they show a better ability to repair strand breaks, through a more potent activation of DNA damage checkpoint mechanisms (Bao, S. *et al*, 2006).

1.8. Targeting glioma stem cells via bone morphogenic proteins

Bone morphogenetic proteins (BMP) belong to the members of the transforming growth factor (TGF)-ß family which have important central roles in controlling cellular proliferation, differentiation, migration, and survival (Letterio *et al*, 2000). These cytokines constitute a highly conserved set of signaling proteins and can be divided into three subgroups: the TGF-ßs, the activins/inhibins, and the bone morphogenetic proteins (BMPs), of which the latter constitutes the largest subfamily. BMPs are 30–35 kD hetero- or homodimeric proteins originally identified by their ability to induce ectopic cartilage and bone formation when injected subdermally in rats (Wozney *et al* 1988). Several studies have demonstrated that these proteins play essential roles during embryonic development (Wall *et al* 1994 and, Kingsley *et al* 1992). For example, BMP2 knockout mice die in utero, BMP7 knockout mice exhibit neonatal lethality, BMP5-deficient mice are viable but exhibit several gross malformations, and BMP6 knockout mice are viable and fertile with only moderate malformations (Zhang *et al* 1996, Jena *et al* 1997, Solloway *et al* 1998). These phenotypes become evident in tissue undergoing morphogenesis, suggesting that BMP signaling is particularly relevant when the cells are being directed toward specific differentiation pathways.

So far more than 20 mammalian BMPs have been identified, but only three type I and three type II receptors capable of binding BMPs have been cloned in mammals (Yamashita *et al* 1996). BMPs induce the heteromeric complex formation between type II and type I receptors. The constitutively active type II kinase activates the

type I receptor, which subsequently propagates the signal downstream by phosphorylating specific BMP receptor-regulated Smads, i.e., Smad1, Smad5, and Smad8 (Yamashita *et al* 1996, Derynck *et al* 1997, Miyazono *et al* 1999). Phosphorylated receptor regulated Smads (R-Smads) form heterocomplexes with the common partner Smad4 (Co-Smad) and translocate to the nucleus, where they participate in the regulation of transcription of target genes (Heldin *et al* 1997).

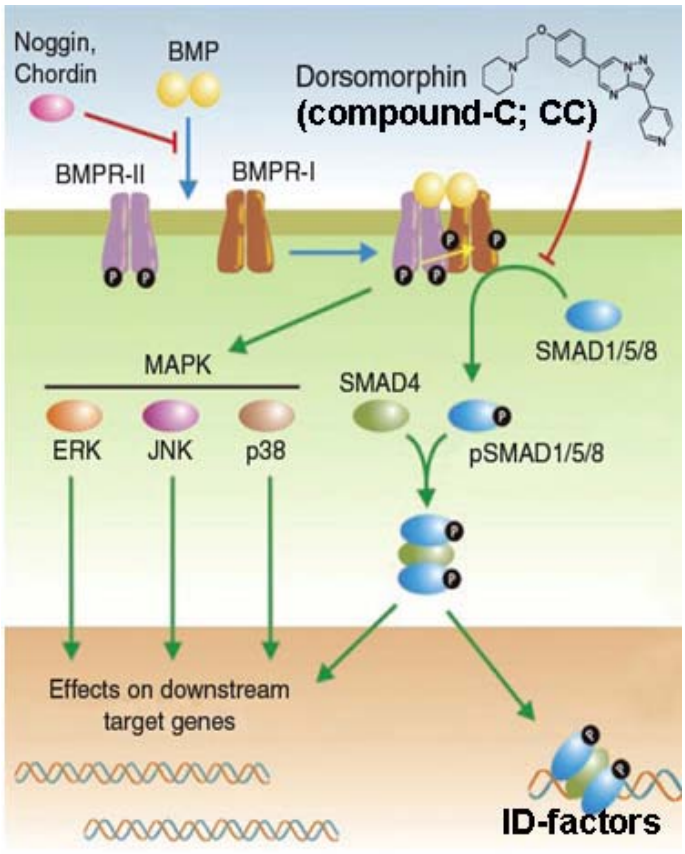


Fig. 1.8. BMP signalling: The BMPs bind to type I and II receptors and facilitate their association. The constitutively active kinase domains of type II receptors phosphorylate type I receptors, and this in turn activates the SMAD signaling pathway through phosphorylation of receptor SMADs (SMAD1, SMAD5 and SMAD8). These associate with co-SMADs (SMAD4) to form a heteromeric complex that translocates to the nucleus and stimulates the expression of a wide range of target genes. BMPs can also signal through SMAD-independent pathways, notably via MAP kinases. Dorsomorphin inhibits BMP signaling through the SMAD pathway, likely by affecting BMPR-I kinase activity. Many of the previously known inhibitors of BMP signaling (such as noggin and chordin) act upstream to sequester BMPs and cannot differentiate SMAD-dependent from SMAD-independent signaling. (Modified from Anderson and Darshan et al 2008).

Neural stem cells express members of the bone morphogenetic protein family and their receptors in the adult SVZ further more they instruct adult neural stem cells to adopt a glial fate, (Lledo PM, 2006 *et al*) BMP antagonist noggin, which is released by ependymal cells of brain, induces glial to neuronal fate (Lim D *et al*, 2000).

Dorsomorphin which is known as small-molecule inhibitor of BMP signaling which has been identified in a screen for compounds that perturb dorsoventral axis formation in zebrafish (Anderson and Darshan *et al* 2008) and found that dorsomorphin selectively inhibits the BMP type I receptors and thus blocks BMP-mediated SMAD1/5/8 phosphorylation and further target gene transcription as shown in Fig.1.8.

Promotion of tumor stem-cell differentiation may be an important strategy for treatment of brain tumor stem cells. Bone morphogenic proteins (BMPs), which normally induce astrocyte differentiation from normal neural precursors, have been shown to promote glioblastoma cell differentiation in vitro and in vivo. Most importantly, recombinant BMPs induce the suppression of glioblastoma tumorigenicity in vivo, possibly though promotion of the differentiation cancer stem cells in the tumor (Piccirillo, S.G., *et al* 2006).
Recently, our group has discovered that endogenous precursor cells are attracted to experimental brain tumors and mediate an anti-tumorigenic effect by inducing glioma cell death in the bulk tumor (Glass, R. *et al*, 2005 and Walzlein, J.H. *et al* 2008). However in these initial studies it was not yet shown that the endogenous neural precursor cells exert an additional further anti-tumorigenic effect by targeting glioma stem cells. In one part of my current work I provide evidence that neural precursor cells suppress glioma initiating cells via release of BMP7.

2. Molecular mechanisms of glioma growth control

One major goal of my studies was the characterisation of cell-cell interactions between gliomas and the tumor microenvironment. In addition to my work on the interaction between glioma, glioma initiating cells and endogenous neural precursors (as described above), I worked on a line of research elucidating some cell autonomous events in glioma which are important for tumor progression. In this part of my thesis I describe a mechanism for ets transcription factor induced pro-tumorigenic signalling in glioma.

2.1. ETS transcription factors and tumors

In general cancer can be defined as a genetic disease and as a consequence of multiple events associated with initiation, promotion and metastatic growth. Cancer also results from the loss of control of cellular homeostasis. Cell homeostasis is the result of the balance between proliferation and cell death, while cellular transformation can be viewed as a loss of relationship between these events. Oncogenes and tumor suppressor genes act as modulators of cell proliferation, while the balance of apoptotic and anti-apoptotic genes controls cell death. All cancer cells acquire similar sets of functional capacities: (1) independence from mitogenic/growth signals; (2) loss of sensitivity to “anti-growth” signals; (3) evade apoptosis; (4) Neo-angiogenic conversion; (5) release from senescence; and (6) invasiveness and metastasis (Hanahan D and Weinberg RA., 2000). One of the goals of molecular biology is to elucidate the mechanisms that contribute to the development and progression of cancer. Such understanding of the molecular basis of cancer will provide new possibilities for: (1) earlier detection as well as better diagnosis and staging of disease with detection of minimal residual disease recurrences and evaluation of response to therapy; (2) prevention; and (3) novel treatment strategies and the increased understanding of ETS-regulated biological pathways will directly impact these areas.

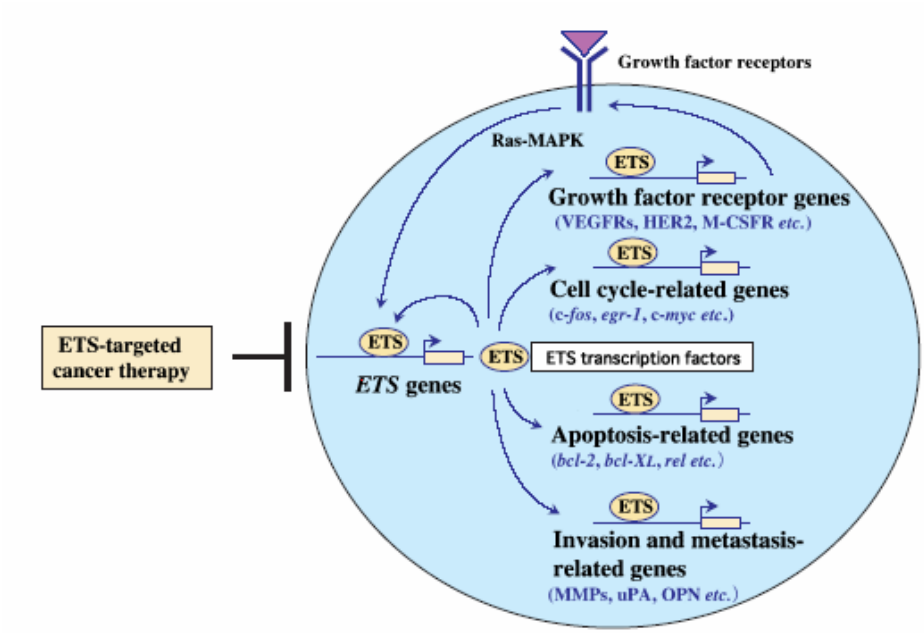


Fig. 2.1: The theoretical feasibility of ETS-targeted cancer therapy. ETS transcription factors are critical for the regulation of expression of several growth factor receptor genes and cell cycle-, apoptosis-, angiogenesis-, and invasion and metastasis-related genes. Thus, ETS-targeted cancer therapy could be a novel approach to inhibit expression of these cancer-related genes. (Adapted from Tsuneyuki Oikawa, Cancer Science, 2004).

As shown in Fig. 2.1. ETS proteins are transcription factors that activate or repress the expression of genes that are involved in various biological processes, including cellular proliferation, differentiation, development, transformation and apoptosis (Oikawa T *et al*, 20004). Identification of target genes that are regulated by a specific transcription factor is one of the most critical areas in understanding the molecular mechanisms that control transcription. Furthermore, identification of target gene promoters for normal and oncogenic transcription factors provides insight into the regulation of genes that are involved in control of normal cell growth, and differentiation, as well as provide information critical to understanding cancer development.

As cancer formation and progression is a complex process determined by several mechanisms that promote cell growth, invasiveness, neo-angiogenesis, and render neoplastic cells resistant to apoptosis. The proto-oncogenic factors ETS are important regulators of such mechanisms as it influences various aspects of cell behaviour by regulating the transcription of specific genes.

The Ets transcription factors regulate many genes associated with tumor invasion, angiogenesis, cell adhesion, and organs development (Dittmer *et al*, 1998). Ets

family of transcription factors comprising more than 30 different members sharing a highly conserved DNA binding motif termed the ETS domain and through this domain they bind to specific purine-rich DNA sequence containing a conserved motif of GGAA/T in the middle of ten base pairs of DNA and depending on which Ets transcription proteins are binding these flanking sequences are variable (Wasylyk *et al*, 1993).

These flanking sequences are variable and there is evidence that they help to determine which Ets proteins will bind (reviewed in Wasylyk *et al*., 1993; Wasylyk and Nordheim, 1997; Watson and Seth, 2000). Ets-1 is the founding member of the Ets family and was initially identified as the protooncogene corresponding to the v-Ets oncogene of the E26 leukemia virus. In humans, Ets-1 is expressed at high levels in proliferating vascular endothelial cells of the embryo and in blood vessels of the adult during angiogenesis (Wernert *et al*., 1992). In the hematopoietic system the analysis of targeted mutants has revealed an essential role for Ets-1 in the differentiation of all lymphoid cells; it was found to be essential for the development of functional NK cells (Barton *et al*., 1998) and for survival and maturation of B and T cell lineages (Bories *et al*., 1995; Muthusamy *et al*., 1995).

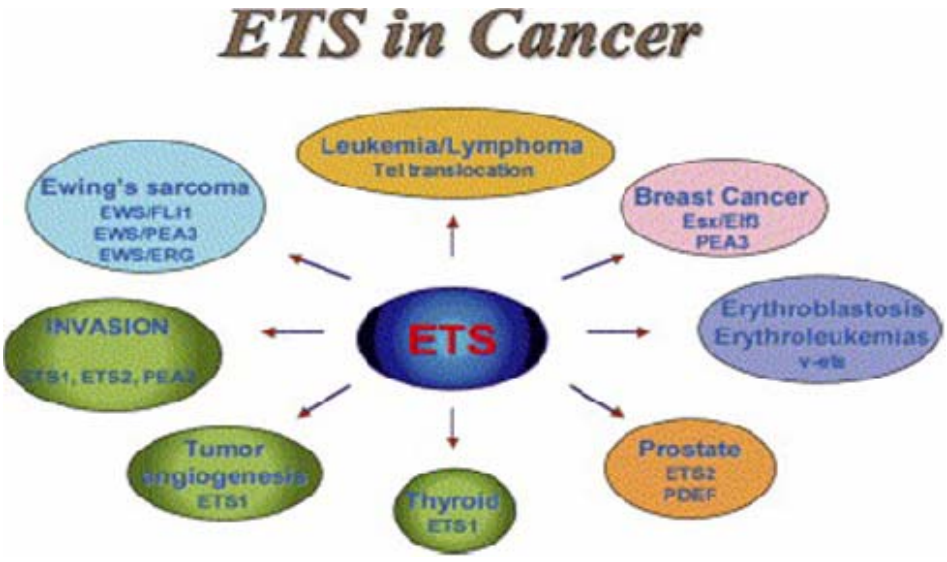


Fig. 2.1.1. ETS transcription factor involvement in cancer. (adapted from European Journal of Cancer, 2005).

The protumorigenic effects of ets transcription factors are long known (Dittmer and Nordheim, 1998) and especially the invasion promoting role of the founding member of the ets family ets1 has

been described in glioma (Kita et al., 2001; Kitange et al., 1999a; Kitange et al., 1999b). The major mechanism underlying oncogenic activities of ets-1 involves transcriptional activation of invasive/proliferative related genes by ets-1 binding to DNA. However, it remained largely unexplored how ets factors promote glioma proliferation (Kitange et al., 1999a).

2.2. Transferrin receptor (TfR)

In this part of my project I investigated the role of ets-transcription factor induced transferrin receptor (TfR) overexpression. TfR overexpression in gliomas is well known (Recht *et al*., 1990), but as yet largely unexplored field of research. I provide evidence that ets-induced TfR expression causes aberrant intracellular iron accumulation, which catalyses the formation of reactive oxygen species (ROS) and subsequently leads to accelerated tumor growth and cytotoxicity. Hence I give here a short introduction to TfR mediated iron accumulation and Iron mediated ROS generation.

Iron (Fe) is an essential requirement for the activity of many metabolic processes. This is because Fe-containing proteins catalyze key reactions involving energy metabolism and DNA synthesis. In fact, without Fe cells are unable to proceed from the G_1 to the S phase of the cell cycle. In addition, Fe appears to play a critical role in the expression and regulation of a number of molecules that control cell cycle progression e.g. p53, GADD45 and WAF1/p21 (Darnell G *et al* 1999 and Gao J *et al* 1999). Cells take up iron by the receptor mediated uptake of transferrin bound iron via its binding to the transferrin receptor (TfR) which is internalised by receptor mediated endocytosis as illustrated in Fig. 2.2. The transferrin receptor is a transmembrane glycoprotein consisting of two 90 kDa subunits and Transferrin (Tf) is ligand for the TfR, which is a member of the family of Fe-binding glycoproteins (Morgan, E. H. *et al*, 1996).

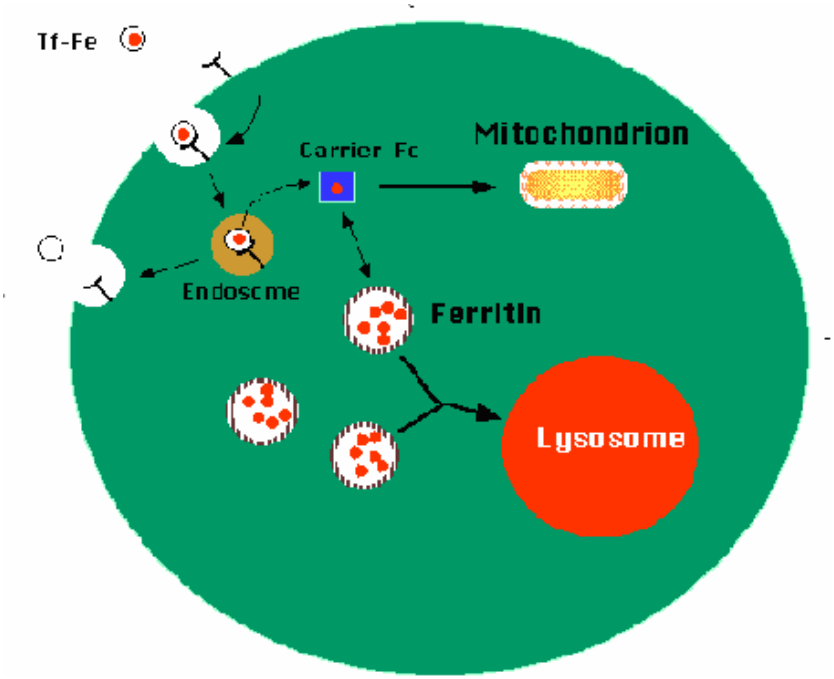


Fig. 2.2: Schematic representation of the TfR internalisation upon binding of Tf to its receptor. Plasma Tf-Fe binds to transferrin receptor on cell surface transferrin receptor with Tf-Fe endocytosed into cell iron regulatory proteins that regulate cellular iron balance by controlling synthesis of apoferritin versus Tf receptor. Iron not required binds to apoferritin to be stored as ferritin in cell.

2.3. Transferrin receptor and its expression in brain

The expression level of the TfR depends on the level of iron supply and rate of cell proliferation. The iron concentration determines TfR synthesis and expression via an iron-responsive element (IRE) in the mRNA of the TfR (Kuhn, L. C. *et al*, 1991, Casey, J. L. *et al*, 1989). This IRE is also found in the mRNA of ferritin, a protein that can store iron (Kuhn, L. C. *et al*, 1991). In cases of low iron concentrations, a so called IRE binding protein stabilises the mRNA of the TfR, which can therefore be translated. The mRNA of ferritin is in low-iron situations less stable and is therefore translated to a lesser extent. Recently, a second TfR (TfR-2) has been identified (Trinder, D. and Baker, E. *et al*, 2003), which does not contain an IRE in its mRNA. TfR-2 is differentially distributed from TfR and has a 25-fold lower affinity for Tf.

The TfR is expressed mainly on hepatocytes, erythrocytes, intestinal cells, monocytes, as well as on endothelial cells of the BBB, transferrin/iron is likely to reach the brain through an endocytotic process at the endothelial cells (Morgan, E.

H. *et al*, 1996, Ponka, P. and Lok, C. N. *et al*, 1999). Furthermore, in the brain the TfR is expressed on choroid plexus epithelial cells and neurons (Moos, T. and Morgan, E. H. *et al*, 2000) and also oligodendrocytes are believed to be the predominant iron-containing cells in the brain and have a special requirement of iron for myelin production, and immature oligodendrocytes express TfR for iron acquisition (Connor, J.R., Menzies, S.L.*et al*, 1996). In normal brain, TfR immunostaining is detected primarily in endothelial and glial cells, whereas neoplastic cells from nearly all brain tumors are found to be TfR-positive (Recht, L., C.O.*et al*, 1990, Prior, R. *et al*, 1990) this is mainly due to the high iron requirements for malignant growth (Ponka, P. and Lok, C. N. *et al*, 1999).

2.4. Transferrin receptors and tumor cells

Most malignant and proliferating cells express high numbers of TfRs as compared to their normal and quiescent counterparts and the first direct evidence of the proliferation-associated expression of TfR was demonstrated in several lymphoblastoid cell lines and activated peripheral blood lymphocytes which have high numbers of transferrin binding sites (Larrick, J.W. *et al*, 1979). Subsequently, numerous reports (Kuhn, L.C. *et al*, 1990, Neckers, L.M. *et al*, 1991) confirmed that virtually all cell lines and normal proliferating cells possess high numbers of TfRs, TfR expression increases upon mitogenic stimulation and diminishes upon terminal differentiation and growth arrest and furthermore anti-TfR antibodies that can block transferrin binding and hence iron uptake inhibit cell proliferation (Trowbridge, I.S. *et al*, 1982). In some tumors like in breast cancers TfR has been discussed to have growth factor like functions (Cavanaugh *et al* 1999) which will support the notion that the TfR is a proliferation-associated marker. The explanation for the proliferation-associated expression of TfR is attributed in part to an increased requirement of iron for synthesis and functioning of numerous iron-containing proteins, in particular ribonucleotide reductase, which is a rate-limiting enzyme in DNA synthesis (Chitambar, C.R. *et al*, 1995). Gliomas are long known to over-express TfR and the extent of TfR expression positively correlates with tumor grading (Recht L *et al* 1990) but the exact role of TfR over expression in glioma is largely unexplored.

2.5. Transcriptional regulation of TfR

Kuhn and coworkers reported the first genomic clone for the transferrin receptor by expression and cloning techniques (Kuhn, L.C. *et al*, 1984). The human TfR gene was found to contain at least 19 exons distributed over 31 kb of DNA and contains a translated region of 2,280 nucleotides in their respective cDNA clones.

TfR gene transcription is activated during cell proliferation (Seiser, C. *et al*, 1993), cell transformation (Beard, P. *et al*, 1991) and differentiation of immature erythroid cells to hemoglobin synthesizing cells (Chan, R.Y.Y. *et al*, 1994, Chan, L.N. *et al*, 1992). On the other hand, gene transcription is comparatively inactive in the quiescent state of the cells, that is during growth arrest and terminal differentiation in the nonerythroid cells (Lok, C.N. *et al*, 1996). TfR gene transcription is believed to be controlled by cellular signals involved in the cell growth and differentiation, resulting in the expression of TfR for iron demand during cell proliferation or heme synthesis. The TfR gene promoter region (fig. 1.10) is GC-rich and contains a TATA box about 30 bp upstream from the known transcriptional start site. A consensus SP-1/GC-rich sequence is located near the TATA box and a sequence as short as 47 bp upstream of the transcriptional start site has been found to be sufficient for driving the basal transcription of the reporter gene as shown by reporter gene assays (Owen, D. *et al*, 1987).

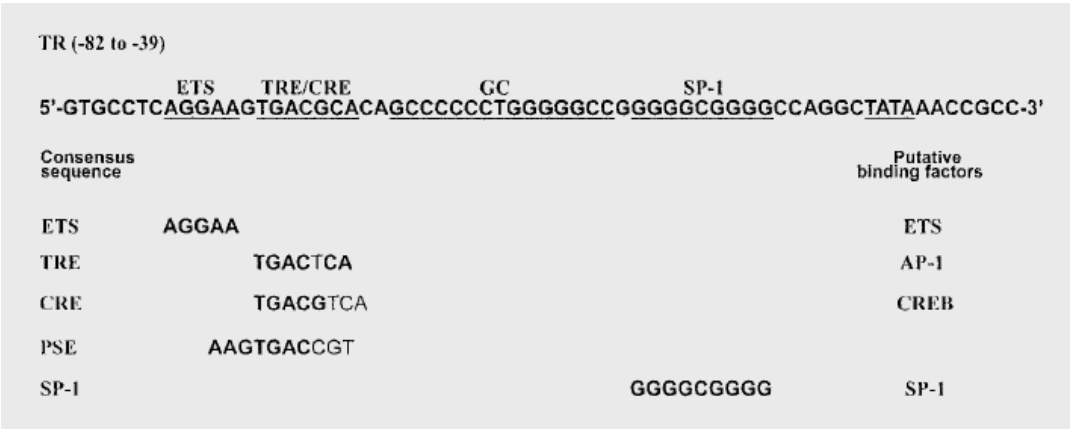


Fig. 2.5. TfR gene promoter region. Part of the TfR promoter region that is critical for its expression. The underlined sequences represent elements similar to certain consensus cisacting elements. Sequence similarity to consensus regulatory cis-elements and putative binding factors are indicated (see text for explanation). Bold typeface identifies bases identical to the TfR gene elements (Sieweke, M.H. et al, 1996, Ouyang, Q. et al, 1993, Roberts, M.R. et al, 1994). CRE = cAMP-responsive element; CREB = CREbinding protein; PSE = proximal sequence element.

One of the most important transcriptional control mechanisms of the TfR is the upregulation of the receptor expression in developing erythroid cells. In an avian erythroblastic cell line, the transcription factor Ets-1 could stimulate the erythroid differentiation and was also found to enhance the TfR promoter activity by 2- to 3-fold via an Ets binding site close to the TRE-like sequence (Sieweke, M.H. *et al*, 1996). Interestingly, overexpression of Maf B, a direct repressor of Ets-1, downregulated the TfR expression and inhibited the erythroid differentiation, without affecting cell growth (Sieweke, M.H. *et al*, 1996). Therefore, an erythroid-specific transcriptional control of the TfR seems to operate. In tumors like in gliomas it is unclear which transcription factors regulate TfR transcription.

2.6. TfR over expression and reactive oxygen species generation

Reactive oxygen species (ROS) have been shown to be associated with a wide variety of pathological phenomena such as carcinogenesis, inflammation, radiation and reperfusion injury. Iron, the most abundant transition metalion in our body, may work as a catalyst for the generation of ROS in pathological conditions. The major reactive oxygen species (ROS) include oxygen radicals such as superoxide (O_2^-) and hydroxyl (.OH), as well as non-radical derivatives of molecular oxygen (O_2), such as hydrogen peroxide (H_2O_2). Whereas O_2^- and H_2O_2 do not exhibit strong reactivity with other bio-molecules, their reaction generates the highly reactive .OH radical, which probably accounts for most of the oxidative damage attributed to ROS (Halliwell & Gutteridge, 1999).

In healthy living cells, one or more of redox regulatory mechanisms are activated in response to a transient increase in intracellular ROS to prevent oxidative stress. A disturbance in the tight balance between ROS production and elimination, either via augmentation of ROS generation or defective/deficient anti-oxidant defenses for their elimination, results in a build up of intracellular ROS and may lead to persistent changes in signal transduction and gene expression (Sauer *et al*., 2001), thereby giving rise to oxidative stress-related pathological states (Burdon, 1995 and Burdon, 1996). In case of tumors like in glioma over expression of transferrin receptors may lead to excessive iron intake into the tumor cells and it is unclear about the fate of free iron in these tumor cells, as from previous reports that free

iron catalyse Fenton reaction and generates reactive oxygen species as shown in the below reaction.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$$

Fenton Reaction: Ferrous Iron(II) is oxidized by hydrogen peroxide to ferric iron(III), a hydroxyl radical and a hydroxyl anion. Iron (III) is then reduced back to iron(II), a peroxide radical and a proton by the same hydrogen peroxide (Halliwell.,1993).

3. Aim of the study

In a first project of my thesis I aim at elucidating the molecular mechanisms of the anti-tumorigenic effect of endogenous neural precursor cells against glioma.

1. To investigate if neural precursors express (anti-tumorigenic) bone morphogenic proteins (BMP) *in vitro* and *in vivo.*

2. To find out if neural precursors attracted towards gliomas maintain BMP expression and if NPCs may (constitutively) release these BMPs.

3. To study if glioma initiating cells may receive BMP signals, and to investigate if BMP signalling has an impact on the tumorigenicity of glioma intitiating cells.

In a second project of my thesis simultaneously I aim to charactirise a cell autonomous protumorigenic effect in glioma mediated by the ets transcription factors.

1. To describe the significance of enhanced transferrin receptor-expression for glioma proliferation
2. To investigate the role of Ets in transcriptional regulation of transferrin receptors and in glioma progression
3. To explore the connection between increased redox signalling and augmented transferrin receptor expression in glioma
4. To identify the potential targets of redox signalling which mediate accelerated glioma proliferation
5. To identify the potential targets of redox signalling, which mediate to accelerated glioma expansion and infiltration.

4. Materials and Methods

4.1. Materials

4.1.1. Devices

CCD camera	Proscan, Germany
Centrifuges	Eppendorf, Germany
Clean bench	Biowizard, USA
Neubauer counting chamber	LaborOptik, Germany
Cryosystem FCS	Leica Microsystems, Germany
Enterprise laser	Coherent, USA
FACSVantage SE flow cytometer	Becton Dickinson, USA
Fluorescence microscope Axioplan	Zeiss
G-Box gel documentation system	Syngene, UK
Gelelectrophoresis device	BioRad, Germany
Gradient cycler	Eppendorf
Incubator	Labotect, Germany
Inverted fluorescence microscope Axiovert 100	Zeiss
Micro-infusion pump	World Precision Instruments, Germany
Microscopes	Zeiss
Nucleofector	Amaxa, Germany
Photometer	Eppendorf
Power Pack 300	BioRad
Scales	Sartorius, Germany
Spectral confocal microscope TCS SP2	Leica, Germany
Speed Vac	Bachofer, Germany
Stereotactic head holder	David Kopf Instruments, USA
Thermocycler T3000	Biometra, Germany
Trans blot SD	BioRad
Victor 1420 Multilabel Counter	Perkin Wallac GmbH, Germany
Vortex	Janke & Kunkel, Germany
Water bath	GFL, Germany

4.1.2. Plastic ware and other material

96-well plates (for DELFIA TUNEL assay)	PerkinElmer, Germany
96-/ 24-/ 6-well plates	TPP, Switzerland
Cuvettes	Eppendorf
Falcon cell culture inserts (0.4 µm) for 6-well	Becton Dickinson
Falcon tubes (15 ml, 30 ml)	TPP
Hyperfilm ECL	Amersham Biosciences, USA
Parafilm	Pechiney Plastic Packaging, USA
Saran wrap	Dow Chemical Co, USA
Tissue culture dishes (60 mm, 30 mm)	TPP
Tissue culture flasks (25 cm^2, 75 cm^2)	TPP

4.1.3. Chemicals

Acrylamide/Bisacrylamide 30 % solution	Sigma, Germany
Agarose	Roth, Germany
Ampicillin	Roche, Germany
APS (ammoniumpersulfate)	Merck, Germany
Aqua Poly/Mount	Polysciences, Inc, USA
BrdU (5-bromo-2-deoxyuridine) labelling reagent	Sigma, Germany
CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)	Amersham Biosciences, Germany
Complete proteinase inhibitor	Roche, Germany
DAPI (4',6-Diamidino-2-Phenylindol-2HCl)	Sigma
DCFH-DA (2'7'dichlorodihydrofluorescin diacetate)	Molecular probes, Germany
DNAse (desoxyribonuclease)	Worthington, USA
dNTPs (desoxyribonucleosidtriphosphate)	Invitrogen, USA
Dorsomorphin (Compund-C)	
dTTP (2'-deoxythymidine 5'-triphosphate)	Roche
ECL Plus (Western Blotting Detection Reagent)	Amersham Biosciences
Ethanol	Roth

FCS (fetal calf serum)	Gibco
G418 (= neomycin)	Gibco
Gel blotting paper	Roth
Glucose	Roth
Glycerol	Sigma
Hybond-P PVDF membrane	Amersham Biosciences
Isopropanol	Roth
Laminin	Invitrogen
L-Glutamine	Biochrom AG
Lipofectamine 2000 transfection reagent	Invitrogen
Matrigel	Becton Dickinson
Methanol	Roth
$MgCl_2$	Invitrogen
PBS (phosphate buffered saline)	Gibco
Penicillin/Streptomycin	Biochrom AG, Germany
PFA (paraformaldehyde)	Merck
Poly-L-Ornithine	Sigma
Polyvinylalcohol	Sigma
Rainbow molecular weight marker	Amersham Biosciences
Sucrose	Merck
TdT (Terminal deoxynucleotidyl Transferase) –buffer	Amersham Biosciences
TEMED (N,N,N',N' Tetramethyl-Ethylen-Diamine)	Amresco, USA
Tris	Roth
Triton X-100	Merck
Trypsin/EDTA (Ethylenediaminetetraacetic acid)	Biochrom AG

4.1.4. Enzymes

SuperScript II Reverse Transcriptase (200 U/µl)	Invitrogen
Taq-Polymerase	Invitrogen
TdT-enzyme (500 U)	Amersham Biosciences

4.1.5. Kits

BCA Protein Assay Kit	Pierce, USA
BMP7 ELISA kit	R&D systems, USA
BrdU Cell Proliferation Assay	Calbiochem, Germany
ChIP-IT™ Express	Active Motif, Belgium
Cell line and Stem cell Nucleofector Kit	Amaxa, Germany
Endo Free Plasmid Maxi Kit	Qiagen, Germany
Dual-Luciferase assay kit	Promega, Germany
QIAprep® Spin Miniprep Kit	Qiagen, Germany
Rneasy Mini Kit	Qiagen, Germany
Site directed mutagenesis kit	Stratagene, Germany

4.1.6. Antibodies

4.1.6.1.Primary antibodies

Tab. 4.1. Overview of applied primary antibodies

Antigen	Host	Dilution	Supplier
5-Bromodeoxyuridine (BrdU)	Rat	1:500	Biozol, Germany
BMP7	Mouse	1:1000	Cell signaling technology, USA
CD133	Mouse	1:200	Milteny Biotec
Transferrin receptor	Mouse	1:400	Dianova, Germany
Ets1 (C-20)	Human	1:500	Santa Cruz Biotechtechnology, USA
Ets-1	Rabbit	1:400	Cell Signaling technology, USA
Glial acidic fibrillary protein (GFAP)	Rabbit	1:500	Dako Cytomation, Denmark
Green fluorescent protein (GFP)	Goat	1:1000	Acris Antibodies, Germany
MAP2	Mouse	1:1000	Sigma-Aldrich, Deisenhofen, Germany
P21	Rabbit	1:100	Santa Cruz

			Biotechtechnology, USA
Psmad1-5-8	Rabbit	1:400	Cell signaling technology, USA
PSA-NCAM	Mouse	1:400	Chemicon, USA
Prominin(CD133)	Mouse	1:10	Milteny biotech, Germany
Ki67	Rabbit	1:400	Novocastra Laboratories Ltd., UK
Smad 1-5-8	Rabbit	1:1000	Santa Cruz Biotechtechnology, USA

4.1.6.2.Secondary antibodies

Tab. 4.2. Overview of applied secondary antibodies

Antigen	Host	Conjugation	Dilution	Supplier
Mouse IgM	Donkey	Rhodamine Red	1:125	Jackson ImmunoResearch Laboratories, USA
Goat IgG	Donkey	Rhodamine Red	1:125	Jackson ImmunoResearch Laboratories
Rabbit IgG	Donkey	FITC	1:125	Jackson ImmunoResearch Laboratories
Rat IgG	Donkey	Biotin	1:125	Jackson ImmunoResearch Laboratories

Substrate	Conjugation	Dilution	Supplier
Streptavidin	HRP	1:10,000	Amersham Biosciences
Streptavidin	Cy5	1:200	Jackson ImmunoResearch Laboratories

4.1.7. Oligonucleotides (PCR primers)

Tab. 4.3. Overview of used oligonucleotides

Name	Sequence (5'→ 3')	Application	Source
m-EBS (forward)	CTCGCGAGCGTACGTGCCTCA CATCGTGACGCACAGCCCCC CTGG	PCR	MWG
m-EBS (reverse)	CCAGGGGGGCTGTGCGTCAC GATGTGAGGCACGTACGCTC GCGAG	PCR	MWG
mutated-AP1/Cre/EBS (forward)	CGTGCCTCACATCGTGTGGTG CAGCCCCCCTG	PCR	MWG
mutated-AP1/Cre/EBS (reverse)	CAGGGGGGCTGCACCACACG ATGTGAGCGACG	PCR	MWG
mutated AP1/Cre (forward)	CGTGCCTCAGGAAGTGTGGT GCAGCCCCCCTG	PCR	MWG
mutated AP1/Cre (reverse)	CAGGGGGGCTGCACCACACT TCCTGAGGCACG	PCR	MWG
hAlk-2 (forward)	ACT ACA GCC TGG AGC ATT GGT AAG	PCR	MWG
hAlk-2 (reverse)	ATC TGC CCA CAG TCC TTC AAG C	PCR	MWG
hAlk-6 (forward)	CGT CCA AAG GTC TTG CGT TG	PCR	MWG
hAlk-6 (reverse)	CCC GAC ACT GAA AAT CTG AGC C	PCR	MWG
hBMPRII (forward)	ACC ATG AAT GGT GTG GCA GGT AGA	PCR	MWG
hBMPRII (reverse)	CTT CAT CAT GGC CAG CTT GTT GCT	PCR	MWG
ß-actin (forward)	CCC TGA AGT ACC CCA TTG AA	PCR	MWG
ß-actin (reverse)	GTG GAC AGT GAG GCC AAG AT	PCR	MWG

4.1.8. Plasmids

Tab. 4.4. Overview of plasmids

Name	Source	Properties
EtsDN construct	H, Sato Japan	Dominant negative form expression of ets
pTRE-tight	M Gossen, MDC, Berlin, Germany	Tetracycline responsive plasmid
pCEP4-IRES-EGFP	Invitrogen	Expresses Enhanced green fluorescent protein
pRc-CMV +TfR	pRc-CMV from invitrogen	Transferrin receptor over expressing construct
pCD-TR1	Kuhn	ORF of TfR

4.1.9. Media and buffer

4.1.9.1.Cell culture media

FCS was inactivated in the water bath for 30 min at 60°C prior to use. All media were purchased from Gibco, USA.

Tab. 4.5. Overview of media and buffer in cell culture

Cell culture medium for GL261 and U373: DMEM/10 % FCS/1 % PGS	10 % FCS 100 U/ml penicillin 100 µg/ml streptomycin 0.2 mM L-glutamine in DMEM
Cell culture medium for electroporation of U373	10 % FCS 0.2 mM L-glutamine in RPMI
Cell culture medium for NPCs and for glioma stem cells(GSCs): NB/B27	20 % B27 100 U/ml penicillin 100 µg/ml streptomycin 10 % L-glutamate 3 mg/ml glucose 20 ng/ml FGF 20 ng/ml EGF in Neurobasal A
Freezing medium for NPCs and GSCs	10 % DMSO in NB/B27
PPD solution	2.5 U/ml papain 250 U/ml DNAse 1 U/ml dispase II (neutral protease) in DMEM 4.5 g/l glucose
Fixans	4 % formaldehyde 0.5 % glutaraldehyde in 0.1 M phosphate buffer

4.1.9.2.Bacteria propagation

Tab.4.6. Medium for bacterial cultures

Luria-Bertani medium (LB)	10 g Bacto-tryptone 5 g Bacto-Yeast extract 5 g NaCl adjust pH to 7.5 with NaOH, autoclave, cool to 55°C and add antibiotics suitable for the expression plasmid

4.1.9.3. Buffers for immunolabelling

Tab. 4.7. Buffers for immunolabelling

TBS	100 mM Tris 150 mM NaCl, pH 7.4
TBS+	% Triton X-100 3 % donkey serum in TBS

4.1.9.4. Buffers for PCR

Tab. 4.8. Buffers for PCR

10 x loading buffer	1.7 % xylen cyanol 1.7 % bromphenolblue 80 % saccharose in H_2O
50 x TAE (Tris-Acetate-EDTA) buffer	242 g Tris-Base 57.1 ml acetic acid 0.5 M EDTA ad 1 l H_2O, pH 8.0

4.1.9.5.Buffers and solutions for Western Blots

Tab. 4.9. Buffers and solutions for Western Blots

Sample buffer	1 % SDS 1 % Triton X-100 Complete proteinase inhibitor in TBS, pH 7.4
10 % APS	100 mg NH_4 persulfate in 1 ml H_2O bidest.
Lower gel buffer	72.7 g Tris 1.6 g SDS ad 400 ml H_2O bidest., pH 8.8
Upper gel buffer	18.2 g Tris 1.2 g SDS ad 300 ml H_2O bidest., pH 6.8

10 x Running buffer	30 g Tris 140 g glycine 10 g SDS ad 1 l H_2O bidest.
Transfer buffer	2.93 g glycine 5.81 g Tris 0.375 g SDS ad 800 ml H_2O and 200 ml methanol
Washing buffer	0.5 % Tween-20 in TBS
Blocking buffer	3 % fat free milk powder in washing buffer

4.1.10. Software

Tab. 4.10. Software

Product name	Supplier
Adobe Photoshop CS	Adobe Systems Inc. USA
analySIS 3.2	Soft Imaging System, Germany
Leica Confocal Software	Leica, USA
Microsoft Office	Microsoft, USA
Image Pro	Media Cybernetics, Silver Spring, MD, USA
Volocity 2.6.1.	Volocity, USA

4.2. Methods

4.2.1. In vivo inoculation of glioma or glioma stem cells into the mouse

4.2.1.1.Animals and Anaesthesia

All animals were sacrificed according to the permit (LaGeSo, 0122/07) given by the Office for Health Protection and Technical Safety of the regional government of Berlin and obeyed the rules laid down in the European Community Council Directive.

Wild type C57BL/6 (Charles River Breeding Laboratories; Schöneiche, Germany) and Nude mice (genetic background athymic Nude-Foxn1, Harlan, Holland) were housed with a 12h light/dark cycle and received food and libitum. All governmental and institutional regulations regarding animal ethics were followed.

Mice were anaesthetised with intraperitonal injections of a 0.1 % xylazyne and 1.5 % ketamine hydrochloride mixture in 0.9 % NaCl. 10 µl of the anaesthetic mixture

was injected per 1 g of mouse body weight. The eyes of the mice were carefully covered with glycerin fat to avoid cornea drying.

4.2.1.2.Glioma cell or GSCs inoculation into the mouse brain

Anaesthetised C57/BL6 mice were immobilized and mounted onto a stereotactic head holder in the flat-skull position. The skin of the skull was dissected with a scalpel blade and the skull surface was disinfected with a 10 % potassium iodide solution. The skull was carefully drilled with a 20 G needle tip at 1 mm anterior and 1.5 mm lateral to the bregma. Then a 1 µl syringe with a blunt tip was inserted to a depth of 4 mm and retracted to a depth of 3 mm from the dural surface. 1 µl of the glioma cell suspension (2 x 10^4 cells/µl) was slowly injected over 2 min into the pre-cast hole and in case of glioma stem cells only 100/µl were taken to inject into mice. The needle was then slowly taken out from the injection canal and the skin was sutured with a surgical sewing cone.

4.2.1.3.BrdU injections

For BrdU labelling experiments, animals received intraperitonal injections of 50 mg of BrdU/kg of body weight at a concentration of 10 mg/ml BrdU in sterile 0.9 % NaCl solution twice daily for three days until 24 h before the glioma cell injection.

4.2.2. Survival study

Glioma stem cells were pre-treated with rBMP7 or NPC-conditioned medium for 5days or left untreated control cells were injected into Nude mice of P25. The application of the cells was performed as described as above**.** Only 100cells/ µl of glioma stem cells were taken for inoculation into mouse.

4.2.3. Tumor size measurement

Tumor xenografts were obtained by subcutaneous (s.c) injection of one million cells of U373wt, U373EtsDn, TfR /U373 Ets Dn into the right and left flanks of female nude mice and two more groups which were injected with U373wt were given DFO and NAC in drinking water.

The tumors were measured in two diameters and the tumor volume (TV) was calculated by the formula TV**:** length (mm) x width^2 (mm)/2= mm^3 (Geran RI *et al*, 1977) over the time course of 2 months.

4.2.4. Paraformaldehyde fixation

The mice were killed by a 10 % ketamin intraperitonal injection and perfused with an intracardiac injection of freshly prepared 4 % paraformaldehyde (PFA) solution (30 ml per animal). The PFA perfusate was replaced by a 0.9 % NaCl solution. After that, the skull was opened and the brain was carefully removed and postfixed overnight in 4 % PFA. Finally, the brains were cryopreserved in 30 % sucrose.

4.2.5. Immunohistochemistry of brain sections (floating sections)

The PFA perfused cryoprotected brains were rapidly frozen in dry ice and mounted onto a sliding microtome. 40 µm thick sections were collected into a CPC (25 % glycerol and 25 % ethylenglycol in 0.05 M phosphate buffer). Before immunolabelling, the sections were washed three times with TBS and subsequently blocked by incubating them in 3 % H_2O_2 (in 50 % methanol) for 30 min at room temperature on a shaker. The sections were again washed three times with TBS and incubated in TBS+ for 30 min for permeabilisation. Then, the sections were incubated for 48 - 72 h at 4°C with relevant primary antibodies. Sections were washed three times with TBS and incubated with the fluorescence conjugated secondary antibodies (1:125) for 3 h at room temperature. After a final wash they were mounted onto microscope glass slides, covered with coverslips and stored at 4°C until used for microscopical analysis.

4.2.6. Cell lines and plasmid constructs

The cDNA-encoding Ets-DN (Kind gift from H. Sato), which lacks a transcription activation domain and corresponds to amino acid residues 306–441 (Kita D *et al*, 2001), was inserted into the pCEP4-IRES-EGFP expression plasmid, allowing bicistronic expression of EtsDN and EGFP. Bulk U373 EtsDn cultures were obtained by stable transfection of that construct into U373 cells with Lipofectamine 2000, Carl. Transfected U373 cells were cultured in the presence of 800µg/ml hygromycin B, and resistant cells were cloned. U373 wild type cells were seeded at clonal density and then cultivated to give rise to a number of clonal U373-WT cultures. A tetracycline inducible system was used to get single clones of etsDn expressing cells of U373. EtsDn-IRES-EGFPwas subcloned in to the pTRE-tight vector (kind gift from M. Gossen). Doxycycline (200ng/ml) was used

to induce the expession of the insert. U373-etsDN cultures were then grown from single clones of the stably transfected cells (as described for U373-WT cells).
Further, I subcloned the ORF for TfR (from pCD-TR1; a kind gift from from L. Kuhn) into a CMV promoter containing vector (Rc-CMV) and then transfected U373 cells constitutively expressing EstDN and WT 1321N1 cells with the TfR construct by using Lipofectamin-2000 Selection of cells stably expressing etsDN+TfR was achieved with hygromycin and G418 (0.6mg/ml). Finally, cultures were grown from single clones of U373-etsDN+TfR cells. All reagents were purchased from Sigma, unless otherwise specified.

4.2.7. Cell culture of glioma cells

The Human glioma U373 and mouse cell line GL261cell line were purchased from the National Cancer Institute, NCI-Frederick (MD, USA). Both U373 and GL261 glioma cells were grown in DMEM/10 % FCS/1 % PGS in T-25/T-75 tissue culture flasks. The mouse GL261 cell line was selected for its isogenity to the mouse strain C57/BL6, which was used for the animal experiments. The medium was changed every two days and cells were passaged when the cell density in the flask reached confluency. Cell cultures were maintained in the incubator at 37°C in a humidified and by 5 % CO_2 conditioned atmosphere.

4.2.8. Cell culture of neural precursor cells

Mice were decapitated and skin and skull were removed. The brain was dissected from the brain stem and transferred into cold PBS/Glucose (4.5 g/l). The cerebellum and olfactory bulb were taken off and cross sections of the brain containing the subventricular zone (SVZ) were made.
The lateral ventricles of the SVZ were microdissected, collected in a 15 ml tube and centrifuged (500 g, 5 min, 4°C). The collected tissue was incubated with occasional mixing in PPD solution (5 ml/animal) for 40 min at 37°C. The tissue was washed three times with PBS to remove the PPD. Cells were plated in 10 cm dishes in NB/B27 (two to three animals per dish). Cultures were incubated at 37°C, 5 % CO_2 and medium was changed on the next day. The cells were cultured until they formed semi-adherent neurospheres. For splitting, NPCs were collected by centrifugation (500 g, 5 min, 4°C) and dissociated by pipette-mixing

for 35 times. The cells were counted and seeded in a clonal density of 500,000 per 10 cm dish in NB/B27.

For adherent cultivation, cells were put on dishes coated with poly-L-ornithine and laminin. These dishes were coated by first incubating them with poly-L-ornithine solution (10µg/ml in H_2O) overnight at room temperature. On the next day they were rinsed twice with sterile H_2O and incubated with a laminin solution (5µg/ml in PBS) overnight at 37°C.

4.2.9. Isolation and Cell culture of Glioma stem cells

Mouse glioma stem cells (Gl261): Bulk cultures of Gl261 cells were grown in NB-27 medium (NPC culturing medium) for at least 4-6 weeks and stem cell like cells were isolated by using Cd133 antibody labeling from FACSorter which were around 0.7-1% and were further enriched to around 90% glioma stem cells.

Human glioma stem cells: NCH421k, these cells were obtained from patients suffering Glioblastoma multiforme and were enriched further to more than 90% CD133 positivity, where as B4 cells are 35% positive for CD133. Both human and glioma stem cells are grown in NB/B27 medium which is the medium I generally culture also neural precursor cells.

4.2.10. Hippocampal cell culture and immunocytochemistry

Hippocampal cultures from E19 Wistar rats were prepared as previously described (Meier J and Grantyn R, 2004) and maintained in B27- and 1% FCS-supplemented Neurobasal medium. The initial cell density was 45,000/cm^2. Tumor cells (2,800/cm^2) were added to the hippocampal cell culture 2 weeks after plating. After 24 hours of tumor cell growth hippocampal neurons were processed for immunocytochemistry. Dendrites were labeled with a mouse monoclonal antibody directed against microtubule-associated proteins (MAP) 2a and 2b and a cyanine (Cy3)-coupled goat-anti mouse antibody. Degenerating neurons were then identified according to the appearance of an abnormal dendrite morphology (length, dendritic swellings and varicosities, fragmentation, as described (Ferrer I, 1999). In addition, the total number of MAP2-positive neurons per viewfield was quantified. Data are presented as means ± s.e.m. obtained from three independent

experiments, each including analysis of at least 2 different cover slips and 5-10 randomly chosen view fields.

4.2.11. Hematoxylin and Eosin staining

The glioma-cell-implanted mice along with control mice brains were postfixed after the perfusion fixation with 4% paraformaldehyde, and cut with a vibratome into 40 mm coral sections. For H & E staining, brain sections were mounted on slide and stained with Harris hematoxylin for 2 min first, and then counterstained with alcoholic eosin.

4.2.12. BrdU proliferation assay

Glioma cells (5 x 10^3/well) were seeded on a poly-L-ornithine-/laminin-coated 96-well plate. The next day, the medium was removed and replaced by fresh medium. Cells were incubated for three days. BrdU label was added to the cells 18 h prior to fixation. The next day, cells were fixed with 200 µl fixative/denaturing solution for 30 min. After that, 100 µl of the anti-BrdU antibody (1:100 in antibody dilution buffer) was added to the cells for one hour at room temperature. Cells were washed three times with 1 x wash buffer and 100 µl of peroxidase goat anti-mouse IgG HRP conjugate was added for 30 min at room temperature. Cells were again washed three times with wash buffer and the plate was entirely flooded with dH_2O. After complete H_2O removal, cells were incubated with 100 µl substrate solution for 15 min at room temperature in the dark. The reaction was stopped with 100 µl of stop solution. The absorbance was measured using a spectrophotometric plate reader at dual wavelengths of 450 – 540 nm.

4.2.13. Immunolabelling

Cells on cover slips were fixed with 4 % PFA for 10 min and then washed three times with PBS. To permeabilise the cell membrane, cells were incubated in TBS+ for 30 min. The primary antibody was applied overnight at 4°C while cells were kept in a wet chamber. The next day, cells were washed three times with PBS and incubated with the fluorescence conjugated secondary antibody for three hours at room temperature. Cells were finally washed three times with PBS and mounted onto microscope glass slides.

4.2.14. Microscopy

4.2.14.1. Fluorescence microscopy

Immunohistochemical preparations were visualized with a fluorescence microscope. Fluorescence microscopy was further used to evaluate transfection rates. Live cultures were analysed for fluorescence using an inverted fluorescence microscope.

4.2.14.2. Confocal microscopy

Confocal microscopy was performed using a spectral confocal microscope. Immunohistochemical preparations were visualized using three different laser channels for FITC (485 nm/535 nm), TRITC (555 nm/575 nm) and Cy5 (650 nm/665 nm). Appropriate gain and black level settings were determined on control tissues stained with secondary antibodies alone. All confocal images were taken with a 40 x magnification objective with a keyhole aperture between 45 µm and 85 µm. Overview images were processed with Photoshop CS and co-localization images were processed with Volocity 2.6.1.

4.2.15. Transfection methods

4.2.15.1. Electroporation (Nucleofection™)

Cells were passaged two to three days before transfection and were growing in their logarithmic growth phase. They were washed once with PBS and harvested by trypsinization. The reaction was stopped by adding serum containing culture medium and cells were centrifuged (800 rpm, 10 min, 4°C). After counting, the required number of cells ($1 \times 10^6 – 5 \times 10^6$ per sample) was centrifuged at 200 x g for 10 min at 4°C. The pellet was resuspended in 100 µl room temperature Nucleofector™ solution and 1 – 5 µg of the relevant DNA was added. The sample was transferred into an Amaxa cuvette, which was inserted into the cuvette holder and the appropriate programme was started. Then, 500 µl of pre-warmed culture medium was added and the sample was transferred into plates, which were pre-incubated with medium. The cells were put back to 37°C and the gene expression was detectable after 6 – 24 h.

4.2.15.2. Lipofectamine transfection

10^6 cells/well were plated one day prior to the experiment in a 6-well plate so that they reached 90 - 95 % confluency at the time of transfection. For one transfection reaction 4 µl of Lipofectamine 2000 was mixed with 250 µl OptiMEM and incubated for 5 min at room temperature. Meanwhile, 4 µg of plasmid DNA was diluted in 250 µl OptiMEM. Then, 250 µl of diluted DNA was added to each vial containing Lipofectamine 2000 and incubated for 30 min. In the meantime, the normal growth medium of the plated cells was exchanged for 2 ml/well of OptiMEM. The transfection mixture of Lipofectamine 2000 and plasmid DNA (500 µl per well) was added to the cells. After six hours, the reagents were exchanged for normal growth medium. After two to three days, cells were trypsinised, pelleted and plated in an appropriate format.

Vial	DNA	Opti-MEM I	Lipofectamine 2000	Opti-MEM I
#1	4µg	250µl	2µl	250µl
#2	4µg	250µl	4µl	250µl
#3	4µg	250µl	8µl	250µl
#4	4µg	250µl	12µl	250µl
#5	4µg	250µl	16µl	250µl
Control	0	250µl	8µl	250µl

Table 4.11. Concentration gradient of Lipofectamine 2000 for a transfection in a 6-well plate

4.2.16. G418 sensitivity test

Prior to the transfection, U373 and GL261 cells were challenged with increasing concentrations of G418, from 0,2 – 2,0mg/ml. The minimal concentration of G418 which was required to kill all the cells after two weeks in culture was taken as a sorting concentration for the selection of stable transfectants. In the case of U373 it was 1,2mg/ml and 600µg/ml for GL261. Briefly, U373 and GL261 cells were trypsinized, centrifuged, counted and plated in 24- well plate at the concentration of $2X10^4$cells/well. Then, they were exposed to increasing concentrations of G418 (see table 2.2.). The medium was changed twice a week. After two weeks in

culture, the minimal concentration of G418 enough to kill all the cells was established. That concentration was later used as selecting concentration of G418 for culturing transfected U373 and GL261.

Control- no G418	Control- no G418	0,2mg/ml G418	0,2mg/ml G418	0,4mg/ml G418	0,4mg/ml G418
0,6mg/ml G418	0,6mg/ml G418	0,8mg/ml G418	0,8mg/ml G418	1,0mg/ml G418	1,0mg/ml G418
1,2mg/ml G418	1,2mg/ml G418	1,4mg/ml G418	1,4mg/ml G418	1,6mg/ml G418	1,6mg/ml G418
1,8mg/ml G418	1,8mg/ml G418	2,0mg/ml G418	2,0mg/ml G418	2,2mg/ml G418	2,2mg/ml G418

Table 4.12. Distribution of increasing concentrations of G418 in a 24- well plate

4.2.17. Cell Cycle Analysis using FACS analyser

For determination of cell cycle progression, the cells were analyzed by flow cytometry. The glioma cells were seeded at a density of $5x10^5$ per 60mm tissue-culture dish. When cultures reached at exponential growth i.e when cells were 80% confluent then cells were collected tripsinization, washed twice with PBS, then fixed with PBS–methanol (1: 2, volume/volume) solution, and stored at 4°C for at least 18 h. After centrifugation, cell pellets were stained with PBS containing 50m g/ml propidium iodide (PI), and 50m g/ml DNase-free RNaseA for 30 min at room temperature in the dark. The samples were analyzed in a Becton-Dickinson system of FACS-Calibur flow cytometer by using CellQuest software. The percentage of cell cycle phases (G0/G1, S, and G2/M) was quantified using a ModFit LT software.

4.2.18. Iron Imaging

Iron measurements were performed using the method by established (Petrat *et al*, 2001). Briefly, Cells plated on glass cover slips were loaded with 20 μm Phen

Green SK, diluted in HBSS, for 15 mints at 37°C and then cells were transfererred to a perfusion chamber on an upright microscope and fixed in the chamber using U-shaped platimum wire. Cells were superfused with Hepes buffer and during the perfusion time 5mM chelator (1, 10-phenantroline, Sigma) was applied and intracellular Fe+2 changes were detected using a cooled CCD camera resulted in an increase in fluorescence. Light source for fluorophore excitation at wavelength of 488nm exitation and 505nm emission. Images and evaluation of iron amount in single cells was determined by the change in fluorescence and processed with conventional software.

4.2.19. ROS measurement

Intracellular ROS production was assessed using 2'7'-dichlorodihydrofluorescin diacetate (DCFH-DA). DCFH-DA is oxidised by ROS within cells to the fluorescent product DCF. Cells seeded in 96 well plate were treated with 50μM of dye and incubated at 37°C for 1hr and the fluorescence was measured using 96 well plate Wallac reader.

4.2.20. Reporter assays

A 455bp fragment of the human TfR promoter region (Owen D and Kuhn LC) was cloned in front of luciferase gene and the resulting construct was transiently transfected into U373 wild type glioma cells using an electroporaor (Amaxa). The activity of pTfR-Luc was compared with mutated TfR promoter constructs in which the Ets respectively the AP1, ATF and CREB sites had been altered by site directed mutagenesis with the following primers from MWG Biotech.

The Dual Luciferase assay was used according to manufacturer's instructions. Briefly, cells were seeded at 10^5 cells per 3.5 cm diameter dish, incubated in medium with or without tet for 48 hours, then washed with PBS and agitated gently for at least 20 minutes with 500 μl passive lysis buffer. The cell lysate was cleared by centrifugation and 20 μl added to 100 μl of luciferase assay reagent in a luminometer tube. The relative light units (RLU) were measured immediately in a Berthold-2000 luminometer. The average of ten RLU measurements taken at 2-second intervals was recorded.

4.2.21. Western blot

4.2.21.1. Sample preparation

Cells were washed two times with ice-cold PBS before the sample buffer was applied (10 µl/cm^2). Cells were scraped with a rubber policeman and the cell lysates were collected in 1.5 ml tubes and incubated on ice for 15 min. Then, the samples were centrifuged at 13,000 rpm for 20 min. The pellet was discarded and the supernatant collected; the protein concentration was determined using the BCA protein assay kit. The protein concentrations of all samples were equalised with sample buffer. Mercaptoethanol (5%) and glycerol (15 %) was added to the samples and they were incubated at 95°C for 15 min.

4.2.21.2. SDS-PAGE

The gels were cast in glass plates. The composition of the gels is shown in tab. 2.12. Gels were loaded with 10 µl molecular weight marker and 20 µl of each sample. Then, electrophoresis was performed at 100 V for 10 min and at 150 V for approximately 45 min.

Tab. 4.13. Composition of a 10 % SDS polyacrylamide gel

Separating gel (lower gel)	
Lower gel buffer	2.5 ml
Acrylamide	3.3 ml
12 % glycerol (in H_2O)	4.17 ml
10 % APS	30 µl
TEMED	15 µl

Stacking gel (upper gel)	
Upper gel buffer	1.25 ml
Acrylamide	750 µl
12 % glycerol (in H_2O)	3.0 ml
10 % APS	20 µl
TEMED	10 µl

4.2.16.2. Semi-dry transblotting

Gels were carefully removed from the glass plates and equilibrated in transfer buffer for 10 min. Meanwhile, a PVDF membrane was activated by incubation in methanol for 5 min and afterwards equilibrated in transfer buffer for 5 min. At the same time, blotting paper was soaked in transfer buffer and placed on the lower electrode (anode). The PVDF membrane and the separating gel were placed between two pieces of moist blotting paper. The sandwich was covered with the upper electrode (cathode) and blotted at 15 V for 60 min.

4.2.21.3. Immunoblotting

The membrane was incubated in methanol for 5 min, washed once in TBS for 5 min and then blocked with blocking buffer for 30 min. The primary antibody was then added in blocking buffer for an overnight incubation at 4°C on a shaker. On the next day, the membrane was washed three times in washing buffer and incubated with the HRP conjugated secondary antibody for one hour at room temperature. After three more washing steps, each for 20 min, the ECL reagent was applied for 5 min. The membrane was dried from excess ECL reagent and placed on a transparent foil. It was exposed in a film cassette to ECL films (in the dark room) for various time periods (e.g.15 s, 30 s, 1 min, 5 min) and the films were developed in the film developing machine.

4.2.22. Oxyblot and detection of oxidised proteins

To identify carbonyl groups that are introduced into the amino acid side chain after oxidative modification of proteins, oxyblot analysis was performed. The derivative that is produced by reaction with 2, 4-dinitrophenylhydrazine (DNPH) was immunodetected by an antibody specific to the attached DNP moiety of proteins using a commercial kit. Briefly, the electrophoresis was performed in the same way as described earlier (Castegna A *et al*, 2002), and the gels were transferred to a nitrocellulose membrane using a semidry transfer system. Membranes were then blocked and incubated with a rabbit anti-DNP antibody (1:150) for 1 hour at room temperature. The secondary antibody incubation was performed using horseradish peroxidase (HRP)–conjugated anti-rabbit IgG (1:300) for 1 hour at room temperature. The immunoreactivity was visualized by enhanced

chemiluminescence using commercial reagents. The kit used for the oxyblot analysis is sensitive to detect as little as 10 femtomoles of dinitrophenyl residues.

4.2.23. Gelatin zymography

Activity of gelatinases (MMPs) was analyzed with the gelatin zymography procedure (Heussen and Dowdle 1980). Briefly, conditioned media from organotypical brain slice cultures or cell cultures or whole slice cultures were mixed with sample buffer, and the samples were loaded on 7,5% SDS-PAGE containing 1% gelatin (table 2.3). After electrophoresis (4°C, 90V), the gel was washed two times 15min in 2,5% Tx-100 washing solution, and incubated overnight in developing buffer. Next, the gel was stained for 30min in 0,5% Coomassie blue solution and then destained in 40% methanol and 10% acetic acid. The gel was wrapped between two foils of cellophane and dried in the gel dryer for 1h. Enzymatic activity resulted in gelatin degradation, which was directly visible as clear bands on the dark blue background. The surface of these clear bands directly correlated to the amount of the active gelatinase. The different gelatinase types were identified by their corresponding molecular weights.

	7,5%PAAG, 1%Gelatin	4% (loading) gel
dd H_2O	4,35ml	6,10ml
20mg/ml gelatin	0,5ml	-
30% Acrylamide	2,5ml	1,3ml
1,5M TRIS HCl, pH 8,8	2,5ml	-
0,5MTRIS HCl, pH 6,8	-	2,5ml
10% SDS	0,1ml	0,1ml
10% APS	0,1ml	0,1ml
TEMED	35µl	35µl

Table 4.14. Composition of 1% Gelatin 7,5% SDS Polyacrylamide gels

4.2.24. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (chIP) assays were carried out using a commercially available kit the chIP-IT™ Express (Active Motif) according to the manufacturer's instructions. One 30-cm dish of 80% confluent wtU373 cells and U373 cells stably expressing etsDN or etsDN + TfR was used for one immunoprecipitation (IP) reaction. Cross-linking of DNA proteins was induced by

an addition of formaldehyde (1% final concentration) directly to the culture medium for 10 min at 37°C. Cells were lysed and DNA in the supernatant was sheared by enzymatic digestion for 10 min at 37°C. An aliquot of the digested chromatin sample ("input") was removed for polymerase chain reaction (PCR) analysis, and the remainder was used for IPs. Immunoprecipitation was performed with 1 µg of Ets1 (C-20) antibody or normal goat IgG that had been precoated onto Protein G-magnetic Sepharose beads, and reactions were incubated for 4 h with constant rotation at 4°C. Protein G beads were collected using magnetic stand and protein DNA complexes were eluted from the beads followed by a cross-link reversal step. DNA from each IP reaction (3 µl) was used for PCR with a primer pair to amplify the 110-bp region of interest of human TfR gene. Input or immunoprecipitated DNA was amplified by PCR (94°C, 20 s; 55°C, 30 s; and 72°C, 30 s) for 35 cycles. In parallel, chromatin was precipitated with positive control antibody (RNA polII) and negative control IgG from the chIP-IT™ control kit (Active Motif); then, input and immunoprecipitated DNA was amplified by 35-cycle PCR with human-specific positive GAPDH primers under recommended conditions, generating 166-bp products. All PCR products were resolved in 1.5% agarose gels.

4.2.25. Identification of mRNA transcripts

4.2.25.1. RNA isolation (RNeasy Mini Kit)

The cell pellet of glioma was disrupted and the lysate was homogenized in 600 µl RLT buffer. After centrifugation (14,000 rpm, 3 min, 4°C) 600 µl of 70 % ethanol was added to the supernatant. The sample was applied to a RNeasy mini column placed in a 2 ml tube and centrifuged at 10,000 rpm, 15 s, 4°C. The flow-through was discarded and 700 µl of buffer RW1 was added to the column. The tube was again centrifuged and transferred into a new collection tube. 500 µl of RPE buffer was added twice onto the column to wash the sample. After transfer of the column to a new 1.5 ml tube, 40 µl of RNase-free water was added onto the membrane. After 5 min incubation the tube was centrifuged at 10,000 rpm for 1 min to elute the DNA. Finally, the RNA concentration was quantified using a photometer.

4.2.25.2. Reverse transcription (RT) – PCR

To obtain cDNA, which can be used for a PCR reaction, RT-PCR was performed. Therefore the following mixture was prepared:

Oligo-dT	1 µl
dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP)	1 µl
Total RNA	1 µg
DEPC-H_2O	ad 20 µl

This was heated up to 65°C for 5 min and quickly chilled on ice. Then, the following mixture was added:

5 x First-Strand buffer	4 µl
DTT (0.1 M)	2 µl
RNase OUT Recombinant ribonuclease inhibitor (49 u/µl)	1 µl

The mixture was incubated at 42°C for 2 min and 1 µl Superscript II (200 U) was added. Hereon, another incubation at 42°C followed for 50 min and the reaction was inactivated at 70°C for 15 min. The cDNA concentration was quantified using a photometer.

4.2.25.3. Polymerase chain reaction (PCR)

To amplify certain segments of the yielded DNA, PCR reactions were performed. Therefore the following reaction was mixed:

Tab. 4.15. PCR reaction mixture

10 x PCR buffer	5 µl
$MgCl_2$	1.5 µl
dNTP mix	1 µl
Primer 1	1 µl
Primer 2	1 µl
Taq	0.4 µl
cDNA	5 µl
H_2O	35.1 µl

Tab. 4.16. PCR-temperature profiles and number of cycles

1. Hot start	94°C	1 min
2. Denaturation	94°C	30 s
3. Primer annealing	Annealing temperature	30 s
4. Elongation	72°C	1 min; 35 cycles
5. End	72°C	8 min
6. Pause	4°C	∞

4.2.25.4. Gel electrophoresis of the PCR products

The PCR products were loaded onto an agarose gel in order to visualize them under UV-light using a fluorescence dye. The 3 % agarose gel (in 1 x TAE buffer) was cast and pre-stained in SybrGold (1:10,000) for 40 min in the dark. 10 µl of each PCR product was mixed with 5 µl loading buffer and loaded into the gel slots; a 1 kb DNA ladder was also loaded. Electrophoresis was carried out at a voltage of 90 V for approximately 45 min. The DNA bands were visualized using the gel documentation facility G-Box.

4.2.25.5. Real time PCR

Quantitative RT-PCR reactions were run in triplicate using Brilliant® SYBR® Green QPCR Core Reagent Kit. SYBR Green dye binds to any PCR product, Fluorescent emission was recorded in real-time (Chromo 4 Four-Color Real-Time PCR Detector**,** MJ Research, Bio-Rad). Gene expression profiling was completed using the comparative Ct method of relative quantification. Relative RNA quantities were normalized to two endogenous controls, GAPDH and 18S ribosomal RNA (18S rRNA).

4.2.25.6. Statistical analysis

Statistical significance was determined at the $p < 0.05$ level. The results are expressed as mean values ± standard errors of the mean (SEM). Comparisons among the groups were performed with the Student's t test.

5. Results-Part I: Mechanism of anti-tumorigenic effect of endogenous neural precursor cells against glioma

5.1. Neural precursor cell-derived BMP7 inhibit the tumorigenic potential of glioma stem cells

5.2. Neural precursor cells (NPCs) express BMP7 in tumor (glioma) margin

We have established previously, that endogenous neural precursor cells **(NPC)** are attracted to experimental brain tumors (gliomas) and induce tumor cell death (Glass R *et al*, 2005) and our further hypothesis is that NPC exert an even more far-reaching anti-tumorigenic effect by suppressing murine and primary-human CD133 positive glioma stem cells **(GSC)** via release of a soluble factor. A previous study by Vescovi *et al* (Piccirillo, S.G.M., *et al* 2006) showed that bone morphogenic proteins (BMPs) induce the suppression of glioblastoma tumorigenicity *in vivo* and *in vitro* via the differentiation of CD133 positive glioma stem cells. In analogy to the physiological action of BMPs in the brain, which induce NPCs to differentiate into mature glial cells (Alvarez-Buylla, A., *et al* 2004). Here we investigated whether the endogenous neural precursors express BMPs *in vivo* and detected predominantly BMP7 by immunohistochemistry especially in the tumor-margin. In this model, gliomas were induced by injection of murine glioma cell line (GL261 which express fluorescent marker) in to a nestin-GFP transgenic mouse and clearly observed the localisation of BMP7 in NPCs which are very near to the tumor margin area as shown in Fig.5.1. Furthermore, I show that NPCs *in vitro* readily release BMP7 and that GSCs respond to NPC-derived BMP7 by undergoing differentiation.

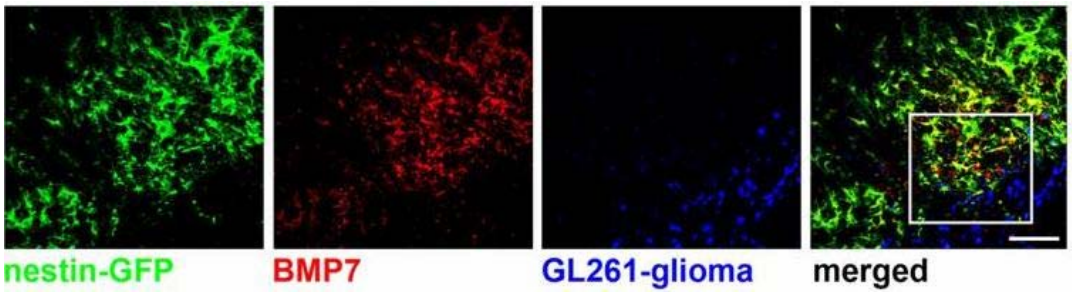


Fig. 5.2. Subventricular zone derived, BMP7-expressing NPC migrate towards glioma.
A transgenic mouse expressing GFP under a modified nestin-promoter was inoculated with syngenic glioma cells (GL261 cells) expressing a fluorescent marker. 14 days after the operation SVZ-derived NPC have accumulated at the experimental tumor. Confocal micrographs giving an

overview on the distribution of BMP7 in NPC at a glioma. Note that in merged fig (extreme right) clearly BMP7 is localised in NPCs close to the tumor margin.Scale:100µm.

5.3. BMP7 expression in the glioma margin is predominantly contributed by migrating NPC

After confirming by immunohistochemistry that BMP7 is expressed in tumor margin, I asked which subtype of NPCs does express BMP7. We found that mainly PSA-NCAM positive NPCs were co-immunolabelled for BMP7, these cells have previously been characterised as the migrating type-A cells (García-Verdugo *et al*, 1998) nestin-GFP transgenic (p30) was inoculated with glioma cells which expressess fluorescent marker and after 14days of operation mouse was sacrificed and stained for nestin-GFP, PSA-NCAM and BMP7 in the glioma margin as shown in Fig.5.3. Using confocal microscopy overlay images were generated by Zeiss ZEN software that showed only the fraction of cells in which two immunofluorescence markers co-localised and detected that PSA-NCAM+/nestin-GFP+ cells predominantly expressed BMP7.

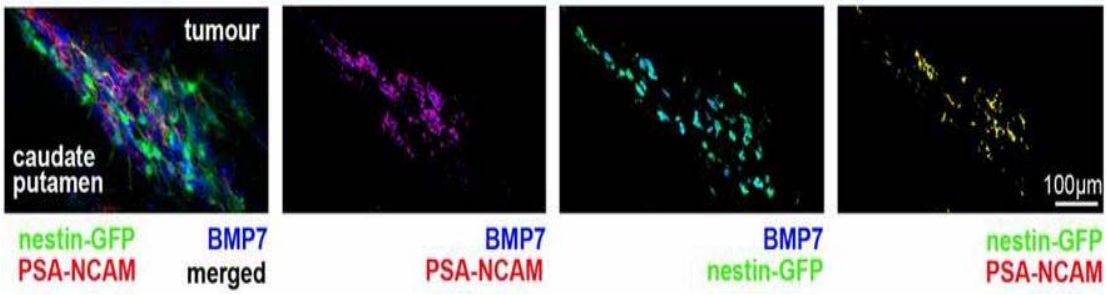


Fig. 5.3. In the glioma margin, only PSA-NCAM-positive NPC predominantly expressess BMP7

Nestin-GFP transgenic mouse (p30) was inoculated with glioma cells (Gl261) which expresses fluorescent marker and after 14 days of operation mouse was sacrificed and processed for immunohistochemistry. Co-localisation of nestin-GFP (green), PSA-NCAM (red) and BMP7(blue) in the glioma margin was investigated by confocal microscopy. Overlay images (generated by Zeiss ZEN software) show only the fraction of cells, in which two immunofluorescence markers co-localise; note that PSA-NCAM+/nestin-GFP+ cells express BMP7. Scale: 80µm.

5.4. Neural precursors in subventricular zone (SVZ) expressess BMPs

After having observed the expression of BMP7 in tumor associated neural precursor cells it was interesting to know whether NPCs in the stem cell niche also

expresses these BMPs. Tissue from nestin-GFP-transgenic mice was immunostained for different BMPs, analysis by confocal microscopy showed that even in normal adult murine brain expresses BMP2, BMP4 and BMP7, especially expression of BMP7 is associated with the NPC which have the migrating capacity i.e. which are PSA-NCAM expressing NPCs. Furthermore, BMP2 and BMP4 were not found in the tumor margin, but could be detected well in the SVZ as seen in Fig.5.4.

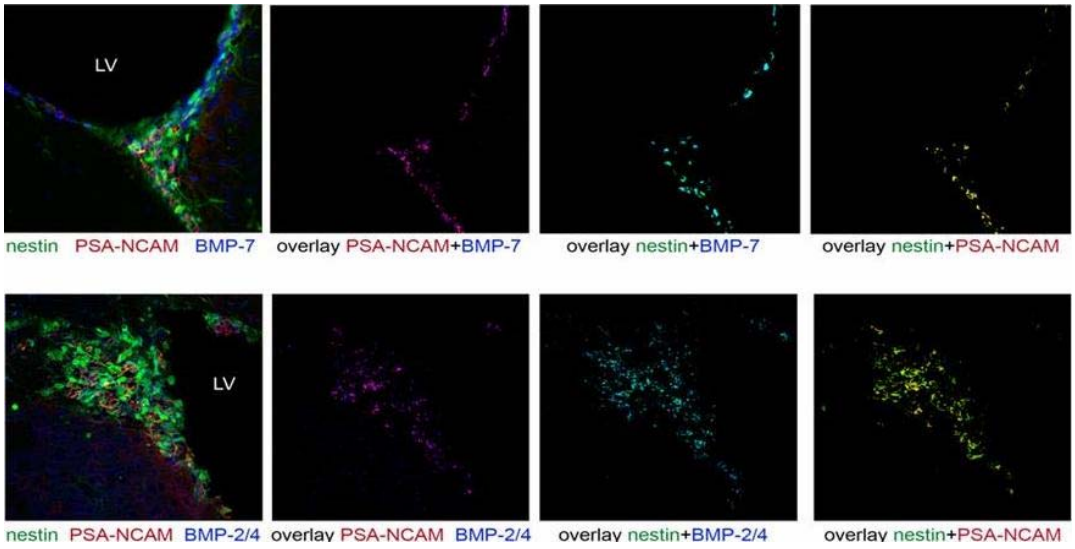


Fig. 5.4. Neural precursors in subventricular zone(SVZ) expresses BMPs
A SVZ explant from a nestin-GFP adult mouse was immunostained for different BMPs and the confocal composite image shows the expression of BMP7 association with PSA-NACM positive NPC(upper panel) and expression of BMP2 and BMP4 co-localised with nestin in the SVZ region (lower panel). Scale bars: 25 µm

5.5. Neurosphere cultures abundantly express BMP7 mRNA

NPCs were isolated from SVZ region of brain from p30 adult mouse and cultivated as neurosheres under standard growth conditions and some NPC cultures were subjected to differentian paradigm (diff-NPC) at the same time glioma cells were also treated with the same conditions as of normal NPC culturing medium and after 3days these cells were pelleted down and isolated mRNA and by using realtime PCR analysed the relative expression of different BMPs like BMP1, 2, 3, 4, 5, 6, 7 and 8b. Vescovi *et al* showed that recombinant BMP4 effectively inhibited glioma stem cell growth but they also reported that recombinant BMP7 is almost as efficient (Piccirillo, S.G.M., *et al* 2006). I discovered that neurospheres

highly expressed mRNA for BMP7 and that NPCs lose BMP7 expression when undergoing differentiation. Glioma cells(GL) and the subfraction of CD133+ glioma initiating cells (GL-CSC) did not significantly express BMPs as shown in Fig.5.5. (n=3).

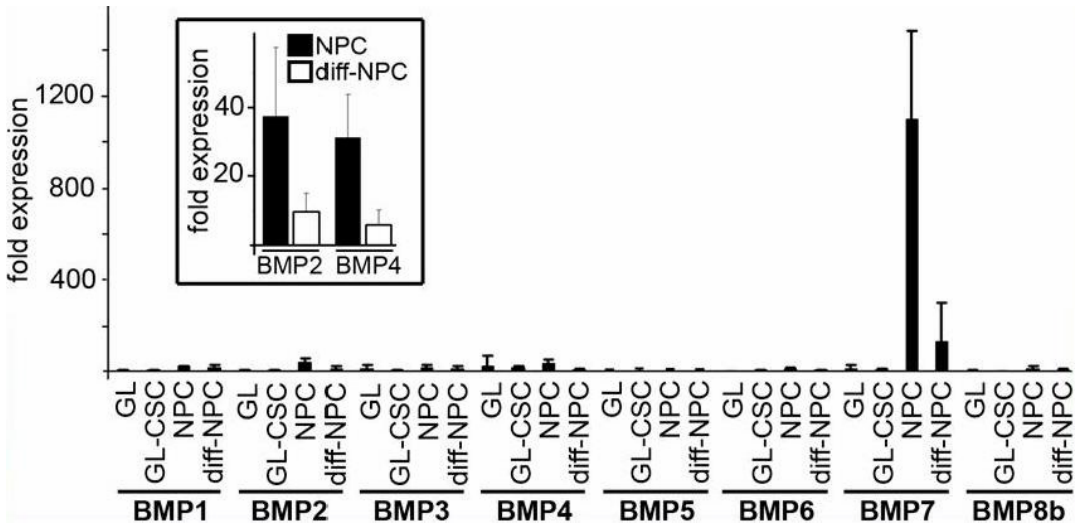


Fig.5.5. Neurospheres highly express BMP7.
NPC were cultivated as neurospheres and some cultures underwent a differentiation paradigm (diff NPC). Likewise glioma cells (GL261WT) were cultured under conditions used for NPCs. GL-CSCs, which are CD133 expresing murine glioma stem cells were also investigated, a range of BMPs was quantified by real-time PCR n=3.

5.6. PSA-NCAM positive NPC neurosphere cultures express and release BMP7 protein

Neurosphere cultures were grown as mentioned above and analysed. Further, PSA-NCAM positive and negative NPCs were isolated by using a FACS sorter, these cells (along with normal bulk NPCs, differentiated NPC and also along with normal primary astrocytes, microglia and neuronal cells) were assayed by ELISA for BMP7 expression.

These assays revealed that BMP7 is much stronger expressed in undifferentiated than in differentiating neurospheres and that BMP7 protein is also much more abundantly in NPCs than in glia or neurons Fig.5.6.A**.** Furthermore, I investigated the conditioned medium from undifferentiated neurospheres and observed that BMP7 is constitutively released from these cultures Fig.5.6.B**.** Furthermore,

immunoblot for BMP7 showed the release of BMP7 from total NPC cell lysates (Fig.5.6. C).

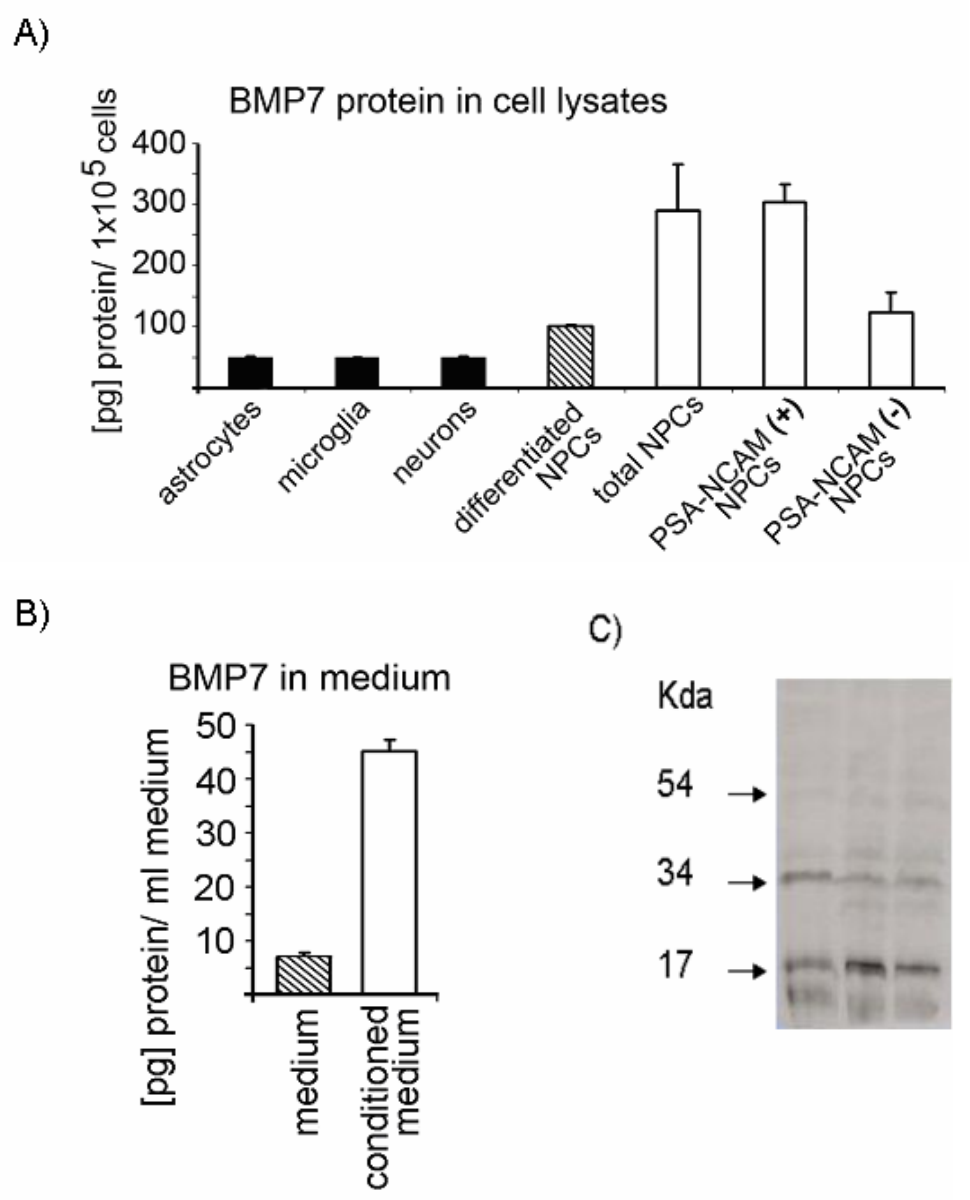


Fig.5.6. BMP7 is predominantly expressed in PSA-NCAM+ cells in vitro and BMP7 is constitutively released.

Cultures of astrocytes, microglia, neurons, neurospheres (total NPCs), differentiated NPCs, and PSA-NCAM+ cells from neurosphere cultures were analysed for BMP7 expression by an ELISA; note that PSA-NCAM+ cells are the predominat cell-type expressing BMP7 in vitro (A), Supernatants from neurosphere cultures were analysed for BMP7 by an ELISA assay(B), (n=3). C) Shows the immunblot for BMP7 from cells lysates of NPCs.

5.7. Glioma stemc cells express receptors for BMPs

To investigate the potential effects of BMPs on glioma stem cells (GSCs) and to determine if glioma initiating cells can receive BMP signals, I used RT-PCR

analysis from cDNA isolated from human glioma stem cells (GSCs), which are more than 95% positive for the glioma stem cells marker CD133. This experiment revealed the expression of receptors for BMP receptor type-1a, type-1b and BMP receptor type II (Fig.5.7.).

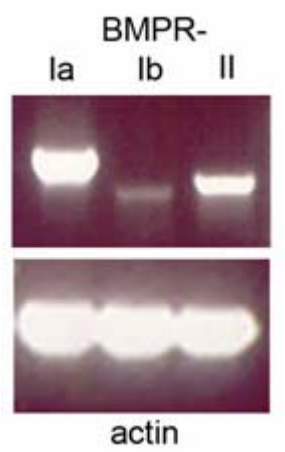


Fig. 5.7. BMP receptor expression analysis by PCR from NCH421k cells
Glioma stem cells (NCH421k) were analysed by RT-PCR for expression of receptors for BMPs like BMPtype I and type II receptors as shown in upper panel BMP type Ia , type Ib and type II receptors are expressed in NCH421k cells, note that type Ia receptor is highly expressed compared to their counter parts typeII receptors, actin loading controls are shown in lower panel.

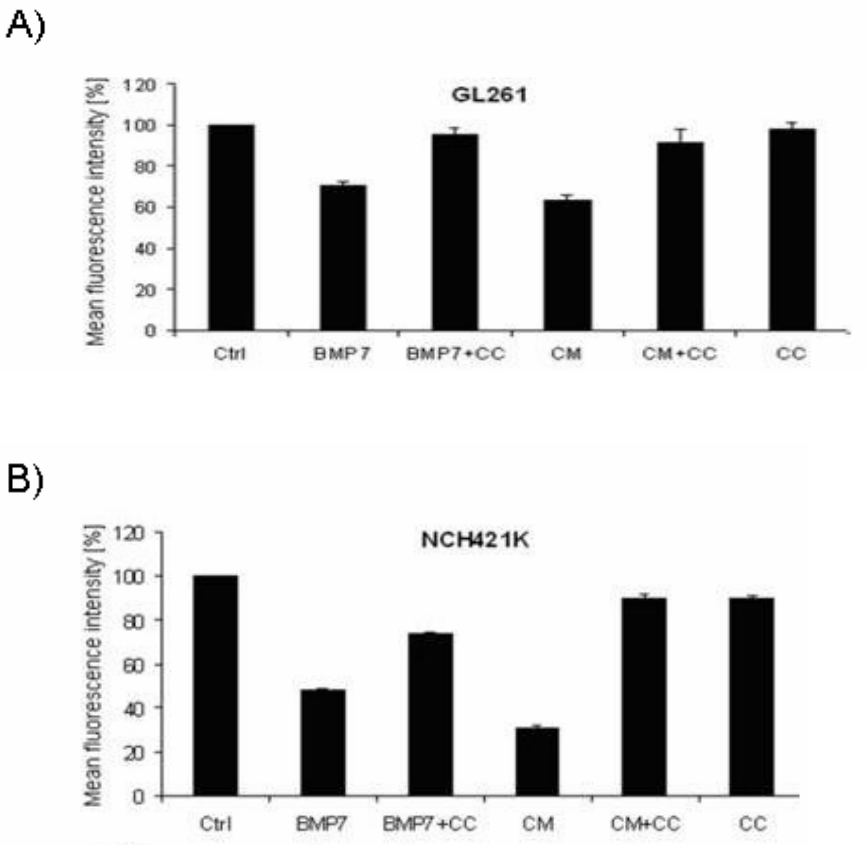
5.8. BMP-release from neurospheres downregulates CD133 expression in Glioma Stem Cells

After detecting the release of BMP7 from NPCs and further identifying the BMP receptors expression in glioma stem cells, I asked whether the conditioned medium from NPCs has any impact on the glioma stem cells. Hence I stimulated different glioma initiating cells NCH421k cells, a human glioblastoma highly enriched for CD133+ cells, B4-cells were from a human glioblastoma and were maintained only for maximally 5 passages in vitro, these cells contained 35% CD133+ cells without prior enrichment and also enriched murine glioma stem cells (Gl261). Cells were left untreated (Ctrl.) or were treated for 5 days with recombinant BMP7 (100ng/ml), neurosphere-conditioned medium (CM) and/or a specific BMP-receptor antagonist (BMPR1a and 1b antagonist) Dorsomorphin (10μM) also called compound-C; CC (Yu, P.B. *et al* 2008)). CD133 expression on the cell surface was quantified by FACS. Interestingly, NPC conditioned medium along with recombinant BMP7 showed 40% reduction in CD133 count in case of murine

glioma stem cell as measured by mean fluorescence intensity, this effect is completely reverted by blocking with dorsomorphin (CC) and treatment with compound-C alone did not show any effect on the expression of CD133 as shown in upper panel of the Fig.5.8.A.

In case of the human glioma initiating cells (NCH421k and B4), NPC conditioned medium showed an even more significant effect as the recombinant BMP7 and reduced the CD133 count to 50% (as shown in B and C of the Fig.5.8.). Note that the addition of recombinant BMP7 to glioma stem cell cultures fully mimicked the effect as of conditioned medium, where a specific BMP-receptor antagonist fully blocked the effect of NPC conditioned medium.

This gave me a clear hint that the released BMPs in the conditioned medium from NPCs might have anti-tumorigenic effect by reducing the CD133 positive glioma stem cells.



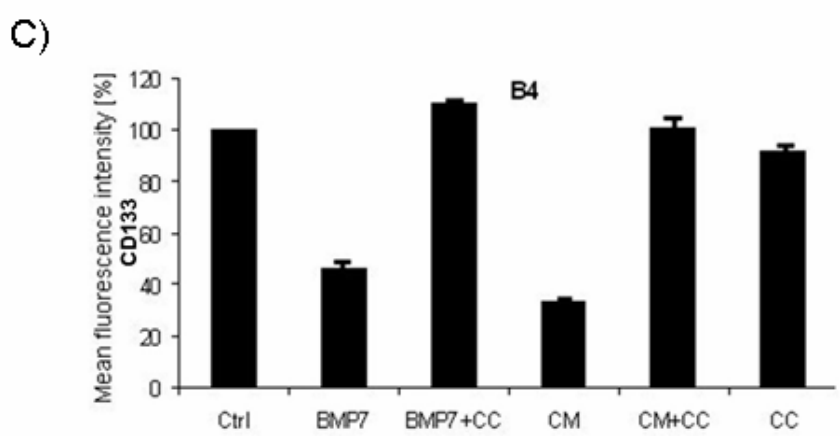


Fig.5.8. BMP-release form neurospheres downregulates CD133 expression in GSCs.
CD133-enriched (> 80%) Gl261, cells are from a mouse glioma cell lines (A) and NCH241K-cells (B) were derived from a human glioblastoma; C) B4-cells were from a human glioblastoma, contained 35% CD133+ cells without prior enrichment and were cultivated for maximally 5 passages. Cells were left untreated (Ctrl.) or were treated for 5 days with recombinant BMP7, NPC neurosphere-conditioned medium (CM) and/or a specific BMP-receptor antagonist compound-C; CC. CD133 expression on the cell surface was quantified by FACS analyser. In case of Gl261 and NCH421k n=3 and in case of B4 n=1, values were represented as meanflourescence intensities.

5.9. Glioma Stem Cells respond to NPC-derived BMP7

Human glioma stem cells, NCH 421k (primary human glioma cells enriched for CD133; i.e. approx. 95% CD133+) respond to exogenous application of 100ng per ml recombinant BMP7 and to the NPC conditioned medium by phosphorylation of smad1/5/8 as detected by western blotting. NCH 421k cells were treated with recombinant BMP7 as wells with NPC conditioned medium (c.m) for 60mints and detected high amounts of phospho Smad-1/5/8(it is detected even after 30 mints of treatment with recombinant BMP7 Fig.5.9.A) compared to untreated control(no treatment). Further, the expression was reversed back to normal levels by using compound-C and treatment with compound-C alone did not show any effect on the expression of phospho-Smad 1/5/8 levels. As loading controls ß-tubulin was used and total Smad 1/5/8 levels were also shown Fig.5.9.B.

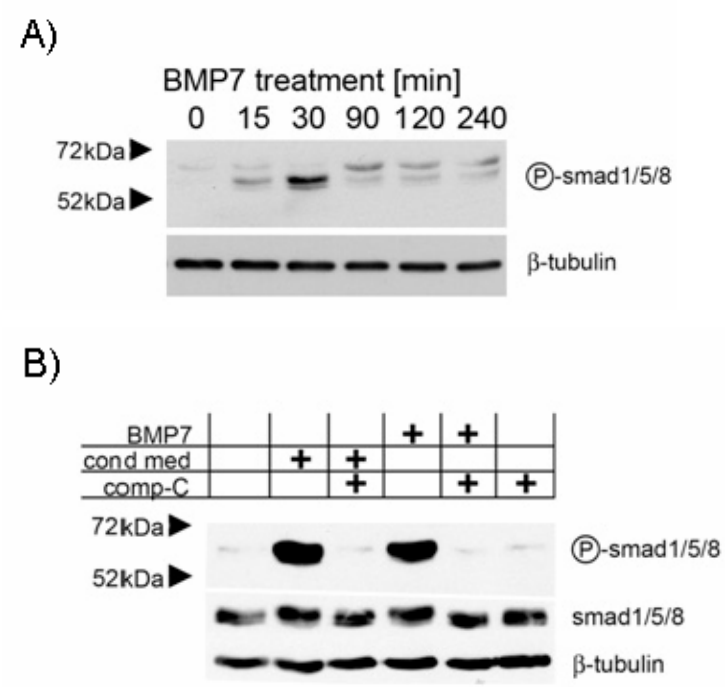


Fig.5.9. BMP7 signalling in Glioma Stem Cells: Western blotting for Smad1/5/8 showing that NCH421k, Human glioma stem cells, respond to NPC conditioned medium and to exogenous application of 100ng of recombinat BMP7(A) by phosphorylation of smad1/5/8; ß-tubulin was used as a loading control along with total Smad1/5/8 levels(B).

5.10. NPC-derived BMP7 downstream signalling in glioma stem cells

Next, I wanted to explore whether NPC-derived BMP7 induced phosphorylation of Smad 1/5/8 also could induce transcriptional changes of target genes. In this regard, the inhibitors of DNA binding proteins (Ids) are considered to be some of the major target genes for Smad-signalling. Thus, glioma stem cells were treated with NPC conditioned medium (CM) and with recombinant BMP7 alone or in the presence of BMP antagonist Dorsomorphin (CC) for 24hrs before preparation of total RNA. The amount of *Id1* and *Id2* mRNA was quantified by real-time RT-PCR. Interestingly, I observed a nearly 25 fold up-regulation of *Id1* mRNA in BMP7 treated as well as NPC conditioned medium treated glioma stem cells compared to Id2 expression which is nearly 7fold up-regulation in comparison to untreated control GSCs and this effect was completely blocked by BMP antagonist Dorsomorphin (CC) (Fig.5.10.).

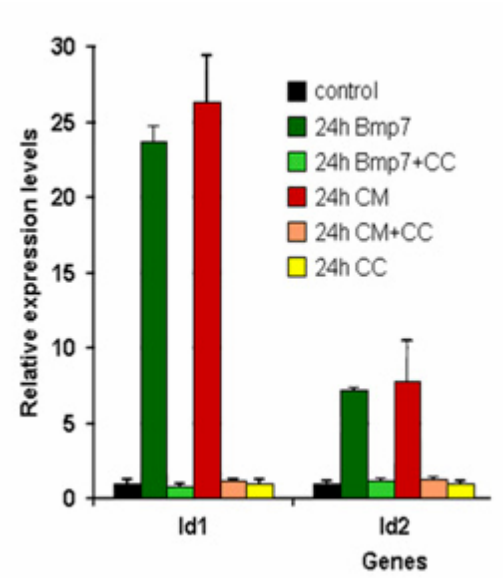


Fig. 5.10. NPC-derived BMP7 downstream signalling in glioma stem cells
Real-time PCR analysis of BMP7 target genes, Id1 and Id2, expression in GSCs treated with BMP7 and NPC conditioned medium (CM) for 24hrs showed the up-regulation of Id1 and Id2; values are normalised to 18S RNA transcript levels and expressed as relative quantification levels.

5.11. BMP7 represses sphere formation of glioma stem cells

NCH421k cells were kept invitro for 5 days and either cultures without additives (control) or treated with recombinant BMP7 (100ng/ml), BMP7 together with the antagonist compound-C or with compound-C alone. Then these NCH421k samples were re-plated in new (additives free) control medium and the ability for sphere formation was observed with different plating densities. After 14 days in control conditions the number of spheres more than >250µm was quantified under a microscope by using a graded plastic dish. Interestingly BMP7 treated cells showed less ability to form spheres compared to controls cells as well as cells blocked with compound-C as shown in Fig.5.11.

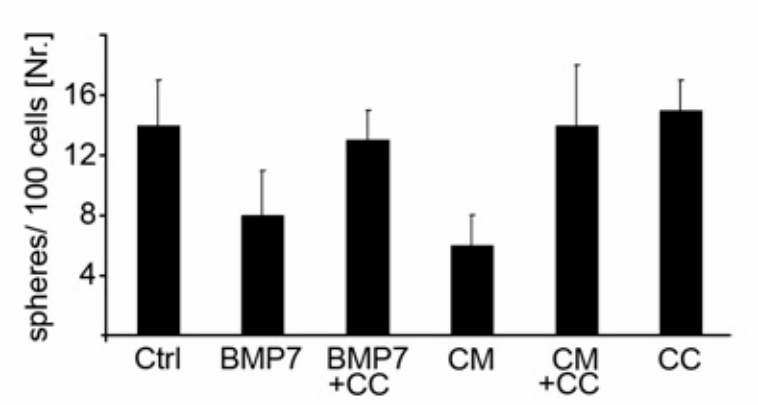


Fig.5.11. BMP7 represses sphere formation of GSCs. NCH421k cells were treated with 100ng/ml of recombinant BMP7 and with other conditions as mentioned above, spheres were dissociated and varying cell numbers were plated in a 24-well plate (containing normal neurosphere medium). After 14 days in vitro the ability for sphere-formation (>250μm) was quantified. Values were represented as number of spheres per well per 100cells.

5.12. NPC derived BMP7 elicits a pro-differentiation effect on glioma stem cells

Glioma stem cells were treated with NPC conditioned medium or with 100ng/ml recombinant BMP7 for 5 days cells, then the cells were observed under phase contrast microscopy. I noticed that the cells treated with C.M. or with BMP7 had a more differentiated morphology as compared to the untreated cells (shown in Fig.5.12.A).

I also observed an increase in the expression of astroglial markers (GFAP) immunoreactivity (Fig.5.12.C) but reduced nestin immunoreactivity after treatment with NPC conditioned medium (Fig.5.12.B) over a period of 5days indicating that the released factors from NPCs i.e. BMPs, which generally induce GFAP expression and eventually promote astrocytic differentiation, show their anti-tumorigenic effect though their pro-differentiation effect on glioma stem cells.

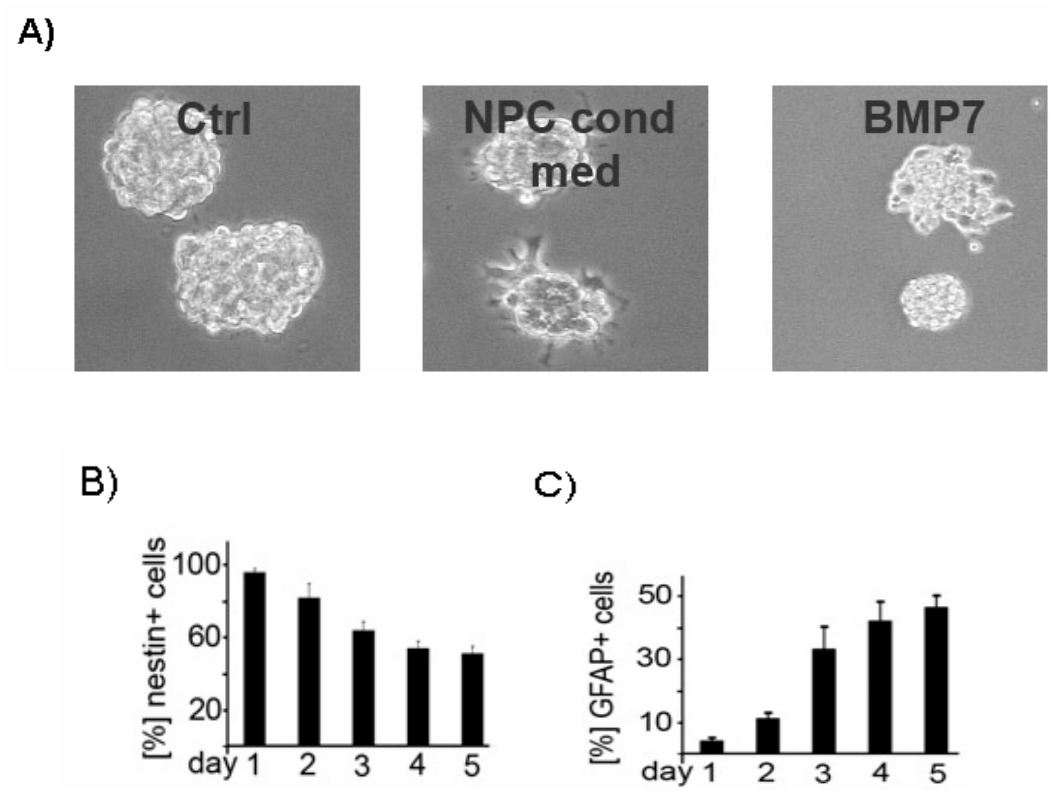


Fig.5.12. Loss of glioma cell identity though differention effect of NPC-derived BMP7

A)Phase contrast images showed that BMP7 and NPC conditioned medium induced a differentiated morphology in glioma stem cell (NCH421k) spheres; BMP7:100ng/ml, B) reduced immunoreactivity of nestin and C) increased GFAP immunoreatctivity after treating glioma stem cells for 5days with NPC conditioned medium, values were represented as %of nestin positive cells as % of GFAP cells.

5.13. BMP7 supressess the proliferation of glioma stem cells and induce cell death

Next, I investigated the effect of BMP7 on glioma stem cells (NCH421K) treated over 5 days on glioma stem cells (NCH421k) which showed a small but significant induction of cell death analysed by cytotoxic assay. The same treatment also triggered a significant loss of proliferation capacity as indicated by a significant decrease in the Ki67 labelling index.

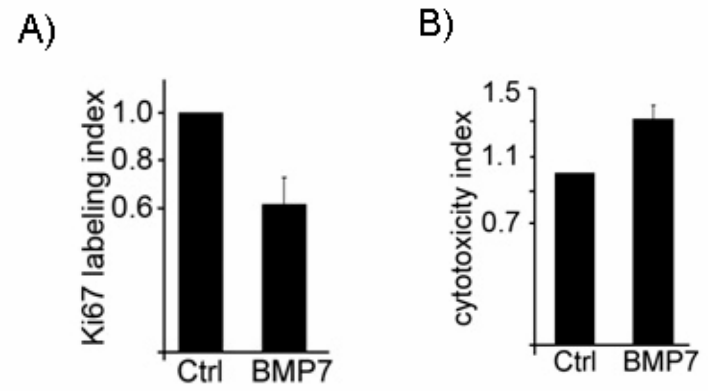


Fig.5.13. Analysis of cytotoxicity and proliferation in BMP7-treated glioma stem cells.

Over 5 day exposure of BMP7 to glioma stem cells (NCH421k) did not significantly induce cell death analysed by cytotoxic assay(B). Conversely, the same treatment triggered significant loss of proliferation capacity as indicated by a significant decrease in the KI67 labelling index(A).

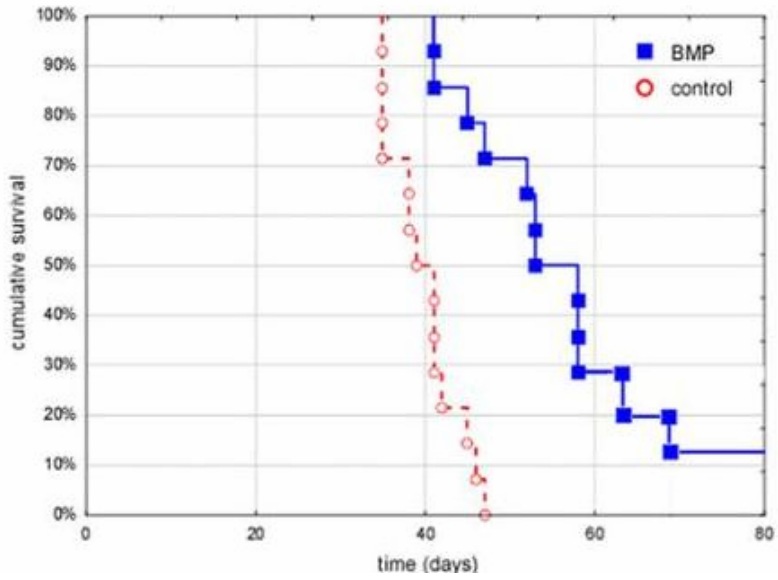
5.14. NPC-derived BMP7 inhibits tumorigenicity of glioma stem cells and prolongs survival in a mouse model

Since NPC-derived BMP attenuated glioma stem cell marker expression, I investigated if NPC conditioned medium and recombinant BMP7 can also interfere with glioma stem cell functions, which are defined by an unlimited capacity for self renewal, maintenance of a cellular hierarchy and strong tumor-initiating ability.

I tested the survival of mice inocculated with two different populations of glioma initiating cells. The GL261 cells were sorted for CD-133 expression (the

population was 80% positive for CD-133). Cells were either treated for five days with recombinant BMP-7 and then inocculated in mice. In comparison to classical tumor cell injections we only injected 100 cells per animal. The second, control group was injected with untreated cells. As shown in Fig.5.14.A the survival of mice inocculated with BMP-7 treated cells was significantly longer as compared to control. In another experiment I investigated if neurosphere conditioned medium would also have an effect on tumor initiation and survival in mice. I added NPC-conditioned medium with or without the BMP receptor antagonist compound-C to NCH421K glioma stem cells, left the cells for five days under these conditions and then implanted 100 cells into brains of immunodeficient mice. I observed that NCH421K cells that had received neurosphere conditioned medium alone had a strongly reduced capacity for tumor-initiation, as compared to human glioma stem cells incubated with NPC-conditioned medium plus compound-C as show in Fig.5.14B. In the group of NCH421K cells exposed to NPC-conditioned medium, six out of twelve mice survived free of symptoms until the end of the study (more thean 240 days), whereas all mice receiving NCH421K cells treated with neurosphere conditioned medium plus compound-C died within an average time of 39 days. All differences in survival time were statistically highly significant ($p<0.001$). Further in Fig.5.14.C showed H and E staining for the same mouse from condition A brain slice after the survival study of which showed, untreated control mouse brain had much more tumor area compared to BMP7 treated one.

A)



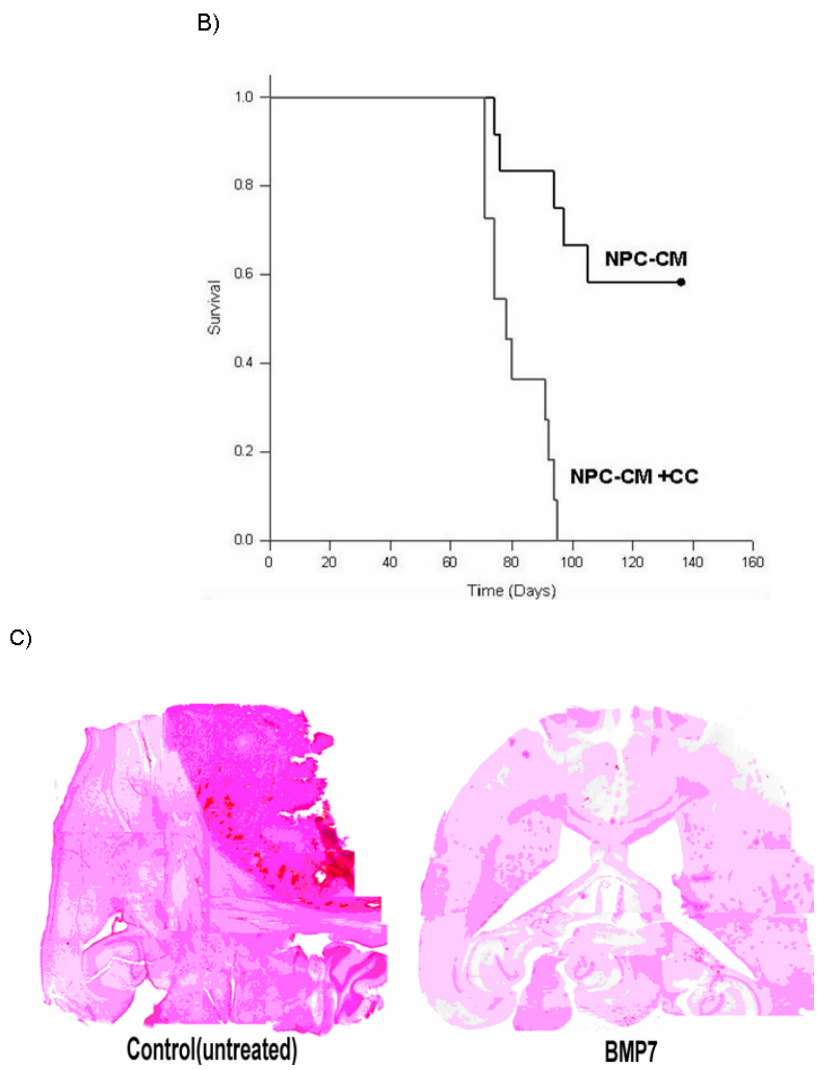


Fig.5.14. NPC-derived BMP7 inhibits tumorigenicity of glioma stem cells and prolongs survival in a mouse model.

Kaplan-Meier survival analysis: GL261 glioma stem cells (>80% positive for CD133) were treated for 5 days with BMP7 or untreated (control) or NCH421k cells with pre-treated NPC-CM or with NPC-CM+ Compound-C (CC). 100 viable glioma stem cells were inoculated in to the caudate putamen of C57/BL6 mice and postoperative survival was monitored; note that pre-treating GSCs with BMP7 or with NPC-CM improves survival (A and B); C shows the H and E staining for the mouse from A.

6. Results part II: Cell autonomous molecular mechanism of ets transcription factor induced pro-tumorigenic signalling in glioma

6.1.Proliferating glioma cells overexpress the Transferrin receptor

Experimental gliomas were induced by injection of GL261 cells (overexpressing GFP) into syngenic mice. A large fraction of the glioma cells massively overexpressed the Transferrin receptor (TfR), compared to normal parenchyma (Fig. 6.1, A and B). In breast cancer TfRhas been discussed to fulfil growth factor-like functions (Cavanaugh *et al*., 1999), therefore we investigated a potential growth promoting effect by TfR in gliomas. Mice bearing a glioma in the caudate putamen were intraperitoneally injected with a single dose of BrdU into the peritoneum 2h before sacrifice. Thereby, I systemically labelled all cells being in S- Phase. In the glioma observed that not all TfR+ cells were in S-phase, but detected that all BrdU+ cells strongly overexpressed TfR.

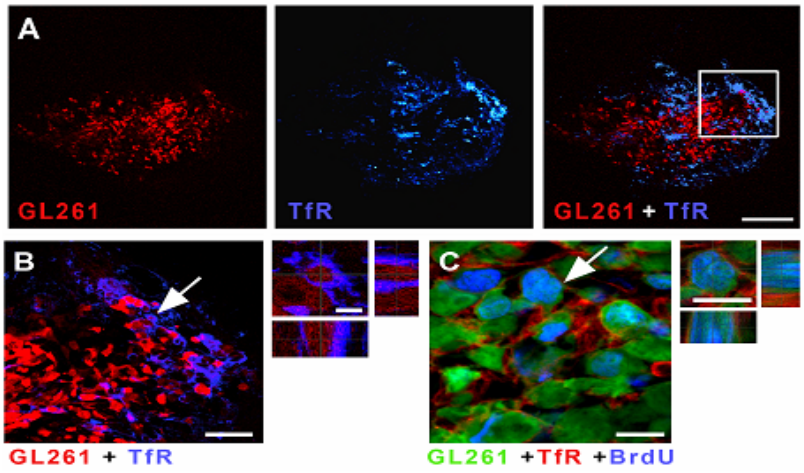


Fig. 6.1 (A) Immunostaining for TfR (blue) in an experimental glioma (red) in mice. TfR is strongly overexpressed only in the glioma. An area marked by the rectangle was magnified in (B) TfR overexpression colocalizes with the glioma cells in the tumor area and on the single cell level; a single cell (highlighted by arrow) was 3D reconstructed cell (insert in B). (C) Strong labelling for TfR (red) in GL261 glioma cells (expressing GFP) having incorporated BrdU (blue), after systemic BrdU administration.

6.2. Transferrin receptor overexpression in glioma is regulated via an ets transcription factor binding site in the TfR promoter

First, we confirmed that TfR is overexpressed in the human glioma cell line U373, compared to the low-malignancy astrocytoma line 1321N1 and to primary cultured astrocytes (Fig.6.2. A). In the same Western blot we show that overexpression of an ets1 dominant negative protein (etsDN; Nakada *et al*., 2001) substantially downregulated TfR in U373 cells. TfR expression was rescued in etsDN cells transfected to overexpress human TfR (Fig.6.2, A). Next, we screened for further transcription factors potentially controlling TfR expression. Therefore, we used a luciferase coupled TfR promoter construct containing an overlapping consensus sequence for binding of AP1, CREB and ATF and also an ets binding site (Marziali *et al*., 2002; Owen and Kuhn, 1987). Mutation of the ets binding site strongly attenuated TfR expression, whereas mutation of the AP1/CRE/ATF site remained largely without effect, arguing that ets transcription factors drive the expression of TfR in glioma (Fig. 6.2, B).

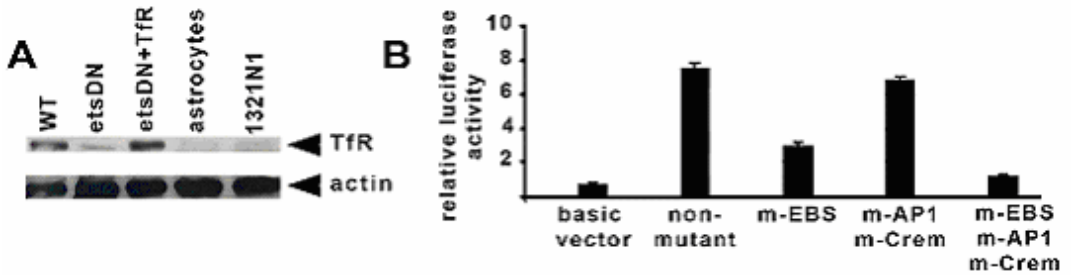


Fig. 6.2. Overexpression of transferrin receptors is regulated by ets transcription factors in glioma

(A) Western blot showing overexpression of TfR in WT U373 glioma vs. primary astrocytes and 1321N1 astrocytes, dominant-negative U373 (etsDN) and etsDN+TfR overexpressing U373 (etsDN+TfR). (B) TfR promoter luciferase-reporter assay in a promoter containing an Ets, Ap1 and Cre binding sites (non-mutant) compared to basic vector. Reporter activity is reduced after mutation of the Ets binding site (m-EBS) but not after mutation of Ap1 and Cre sites (m-Ap1 and m-Crem).

6.3.Trancription factor ets1 directly binds to the promoter region of the TfR gene

A chIP revealed that ets1 directly binds to the promoter region of the TfR gene (Fig. 6.3, A). Ets1 binding is seen only in WT cells (see arrow in Fig. 6.3, A) and is prevented in etsDN-expressing cells. These data show that etsDN overexpression directly blocks ets1 function. Western blotting for a phosphorylated (and therefore hyperactivated) form of ets1 in nuclear and cytosolic fractions of U373 cells (Fig.6.3, B) indicated phosphorylation of ets1 in WT cells, which was attenuated by etsDN expression. However, forced expression of TfR rescued the phosphorylation of ets1 in etsDN-expressing cells, suggesting a positive feedback signal from TfR for ets1 activation.

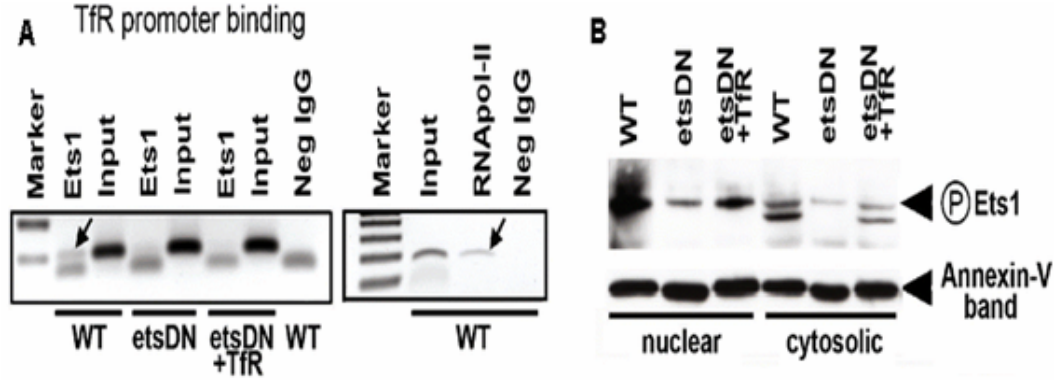


Fig.6.3. Chromatin immunoprecipitation assay showing the binding of ets1 to the promoter region of TfR gene

WT, etsDN, or etsDN + TfR cells were taken for chromatin immunoprecipitation assays, either using an anti-Ets1 (C-20) antibody or an unspecific IgG (Neg IgG) as a control. Total cell lysates were used as a positive control (input). The specific PCR product is a 110-bp sequence of the promoter region of the TfR gene. Positive chIP is indicated by an arrow (A). A control immunoprecipitation and PCR reaction with GAPDH primers was carried out in parallel on WT cells (right panel). B. Western blotting for phosphorylated ets1 on nuclear and cytosolic fractions of WT, etsDN, and etsDN-TfR cells; cross-reactivity of an ets1 antibody with annexin-V was used as a loading control.

6.4. Alteration in expression of transferrin receptors in glioma changed their morphology

The morphology of wild type U373, U373 EtsDn and U373 etsDn+TfR cells cultured on cover slips are compared, wild type and transferrin receptor over expressing cells are exhibited round morphology where as EtsDn cells showed spindular morphology. Further, I observed under time lapse fluorescence microscopy (in 20X magnification for 20mints perfusion) that WT and etsDn+TfR

are morphologically very dynamic, frequently showing membrane expulsions, intrusions and high cell motility. Moreover these showed active lamellipodia and philopodias where the opposite was observed in etsDn cells, these cells seems were much less dynamic and largely remained in a elongated shaped with very little cellular movement and severe changes in plasma membrane morphology.

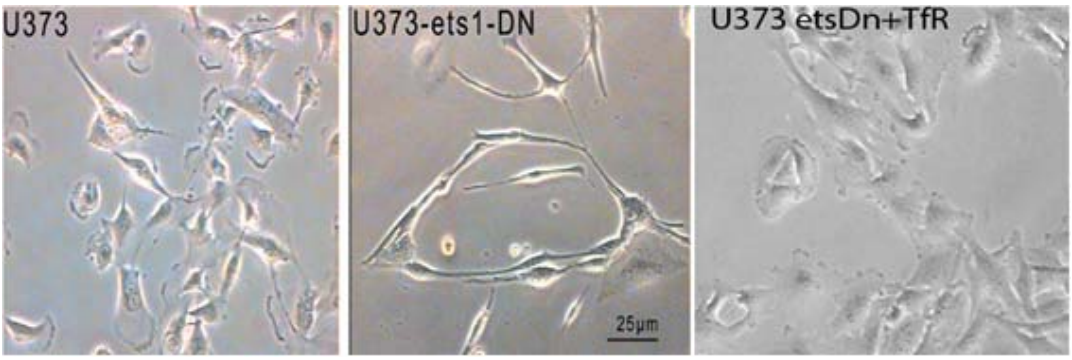


Fig. 6.4. Alteration in expression of transferrin receptors in glioma changed their morphology. Cells of wild type glioma (U373), dominant negative form of ets expressing cells (etsDn) and transferrin receptor over expressing etsDn cells (U373 etsDn+TfR) cultured on coverslips were observed with phase contrast microscope under 20X magnification, where the cells of typical glioma U373 showed active lamellipodia and philopodias with round shape the same observed in U373 etsDn +TfR but this is not observed in etsDn cells.

6.5. Over expression of transferrin receptors in glioma is independent of culture conditions

Further, we observed that TfR expression is also largely independent of the culture conditions and hypoxia mediated Hif1 function. In an additional experimental approach we cocultured etsDN expressing U373 cells together with wild-type cells ensuring that both cell types grow identical conditions, especially with respect to the iron content in the culture. In these cocultures only the wild-type cells overexpressed TfR as shown by immunocytochemistry (Fig.6.5.).

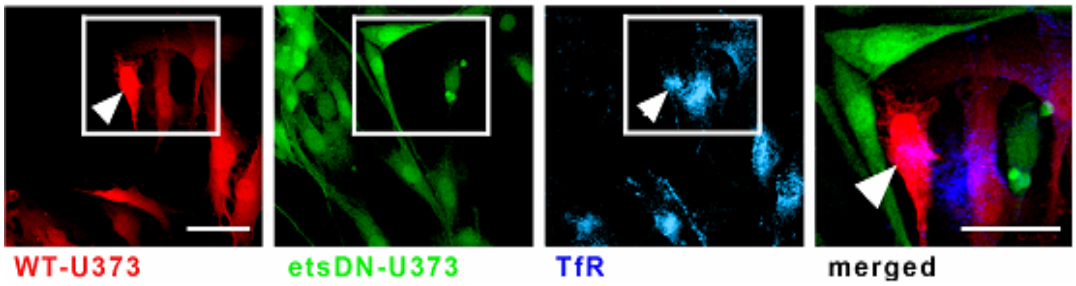


Fig.6.5. TfR expression in glioma in vitro is not altered by the culture conditions
Ds-Red expressing U373 cells strongly immuno-labelled for TfR (arrowhead) while GFP expressing EtsDn cells show low TfR levels in the same culture; note TfR localisation in a magnified image (rectangle).

6.6. TfR expression in glioma in vitro is not altered by hypoxia and Hif1

Under hypoxic conditions or during iron deficiency TfR expression may be induced by the transcription factor Hif1α, which can also bind to the DNA stretch used in our reporter gene assay (see above). However TfR expression did not change under hypoxic conditions (5% Co2, pO2<0.2hpa, for 24h) and after pharmacological inhibition of Hif1α with YC1 (Sun *et al*., 2007) in hypoxic glioma. The corresponding controls show that hypoxia induced and YC1 efficiently blocked accumulation of nuclear Hif1α. Hypoxic U373 cells also had higher Hif1α activity as measured with a luciferase based reporter construct containing Hif1 binding sites.

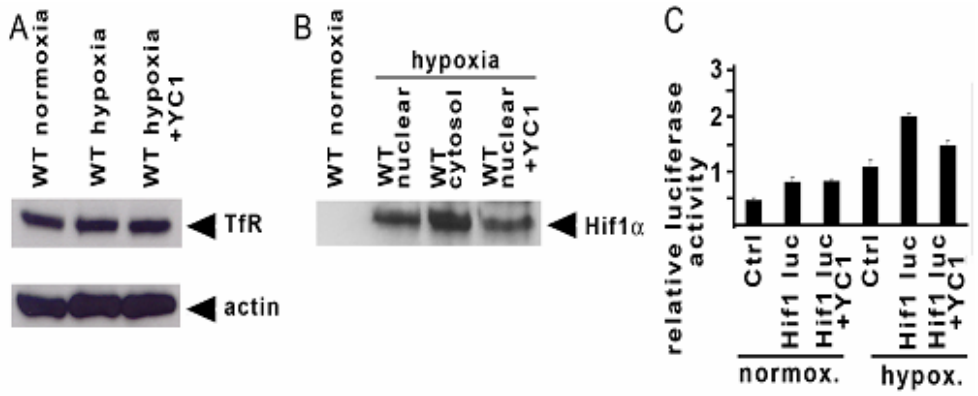


Fig.6.6. TfR expression in glioma in vitro is not altered by hypoxia and Hif1

A) Immunoblot for TfR from U373 WT cells subjected to normoxia or hypoxia for 24hrs, with and without the HIF-1 blocker YC1; note that TfR expression is independent of hypoxia or Hif1. (B) and (C) showing nuclear localisation and activity of HIF-1 in U373 WT cells under normoxia or hypoxia for 24hrs, with and without HIF-1; note that hypoxia enables Hif1 function that can be blocked by YC1.

6.7. TfR expression in glioma in vitro is largely independent of the cellular iron concentration, but depends on the oxidant levels

Western blotting for TfR from U373 cells cultivated under control conditions or together with the iron chelator desferroxamin (DFO; 200μM, for 24h) or the iron donor ferric ammonium citrate (FAC; 50μg/ml, for 24h) revealed unchanged levels of TfR expression (Fig. 6.7. A). Whereas under physiological conditions the expression of TfR is regulated in a complex fashion on the transcriptional (Marziali *et al*., 2002; Sieweke *et al*., 1996) and post-transcriptional (Owen and Kuhn, 1987) level.

Transcription factors besides ets factors and also the cellular iron level had no major effect on TfR expression, but oxidants induced TfR expression. Application of H_2O_2 (50µM, for 24h) further increased TfR expression in U373 WT cells, whereas the anti-oxidant N-acetylcystein (NAC; 200µM for 24h) reduced TfR protein expression (Fig. 6.7.B).

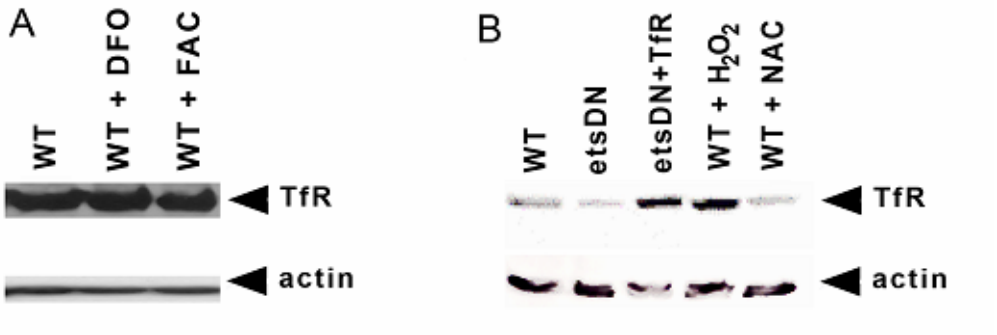


Fig.6.7. TfR expression in glioma in vitro is largely independent of the cellular iron concentration, but depends on the oxidant levels

A) Immunoblot reveals no change of TfR expression in U373 WT cells exposed to the iron chelator deferoxamine (DFO) or the iron donator ferric ammonium citrate (FAC), (B) Western blot showing altered TfR expression upon treatment of U373 WT cells with the oxidant scavenger N-acetyl cysteine (NAC) and the oxidant hydrogen peroxide (H_20_2).

6.8. Ets transcription factors control Transferrin receptor mediated redox signalling

Next, we addressed whether the ets mediated increase in TfR expression had physiological consequences like increased intracellular iron accumulation and increased oxidant production by the labile iron pool (Zecca *et al*., 2004). We clonally expanded U373 glioma cells that were derived from wild type (WT) cells, from cells containing a tetracylin inducible etsDN construct and from cells expressing etsDN plus the human TfR (etsDN+TfR). For iron and reactive oxygen species (ROS) measurements and BrdU assays with these cells we averaged data obtained from three individual clonal lines.

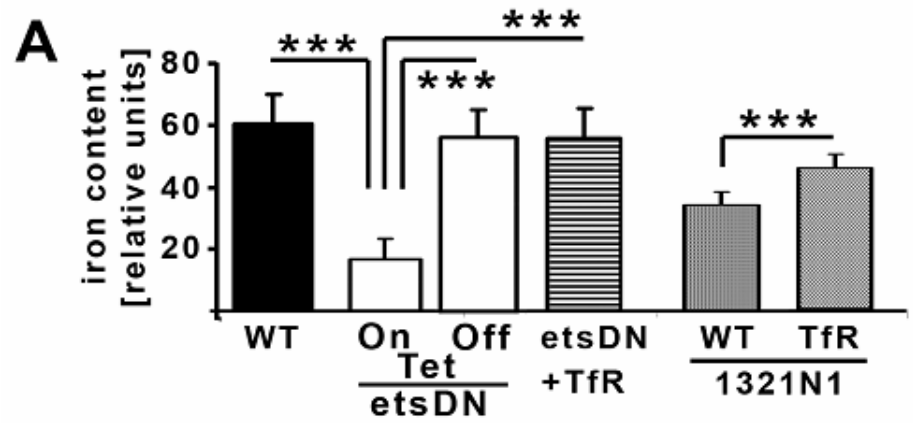
Tetracycyclin induced expression of etsDN (etsDN-TetON) in U373 cells strongly attenuated intracellular iron accumulation, when compared to WT and etsDN cells without tetracyclin (etsDN-TetOff), as measured by iron imaging using PhenGreen-SK as an indicator (Petrat *et al*., 2001). In etsDN+TfR cells intracellular iron accumulation was fully rescued (comparable to WT; Fig. 6.8. A).

EtsDN-TetOn substantially reduced ROS levels in U373 glioma, as compared to WT and etsDN-TetOff, which was evaluated by ROS-imaging using DCFH-DA as an indicator (Mattia *et al*., 1993). Reexpression of TfR in etsDN cells fully restored iron accumulation in glioma (Fig.6.8. B). FACS analysis of DCFH-DA fluorescence in revealed that ROS levels specifically in the estDN-TetOn cells were homogenously diminished throughout the whole cell population (Fig. 6.8. C).

We had shown in our first series of experiments that TfR expression was increased after rising oxidant levels. The finding that increased TfR expression mediates increased oxidant levels offers the possibility that TfR enhances its own expression.

In BrdU incorporation assays proliferation was decreased in etsDN-TetOn cells as compared to WT, etsDN-TetOff and etsDN+TfR cells (Fig. 6.7. D). The proliferation of U373 cells directly depended on iron and ROS levels since DFO and the NAC largely attenuated glioma proliferation (Fig. 6.8. E).

Further, we compared the results in the U373 glioma line with those of the low grade astrocytoma line 1321N1. These cells constitutively had a significantly lower iron content, ROS levels and proliferation rate as compared to U373 cells. Overexpression of TfR in the 1321N1cells increased all three parameters (iron accumulation, oxidant production and excessive growth) as shown in Fig. 6.8.



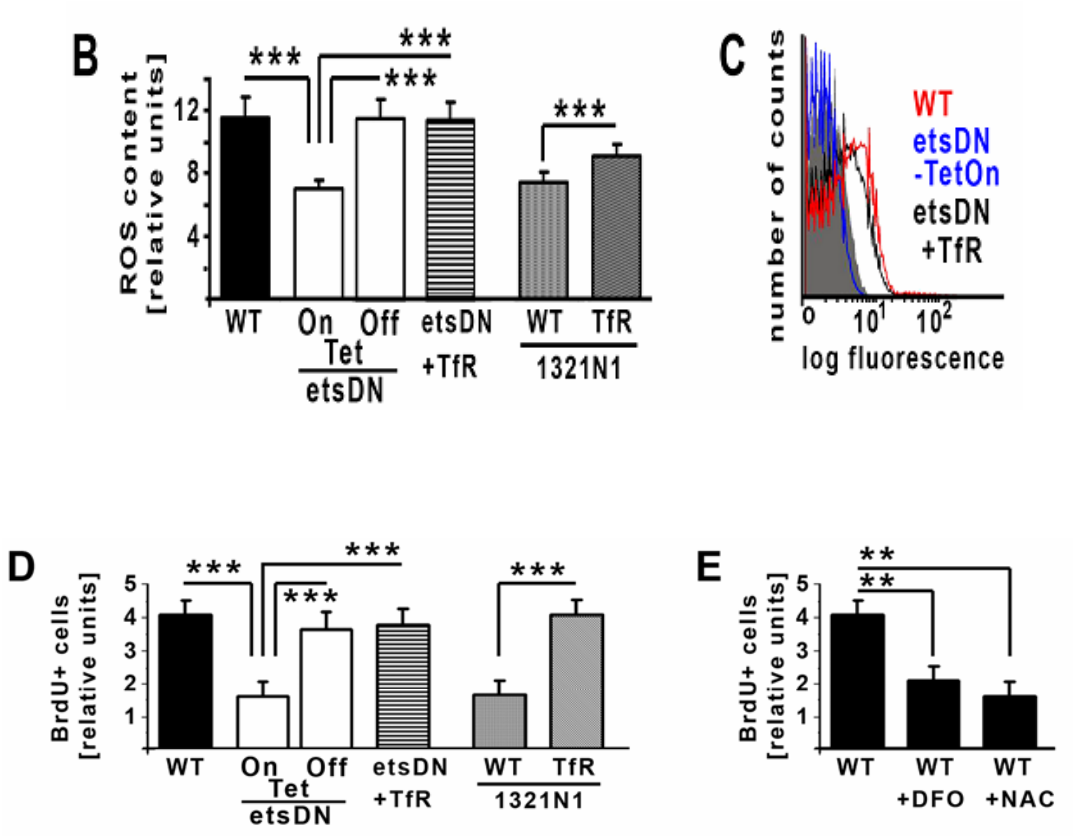


Figure: 6.8. Induction of transferrin receptor mediated redox signalling is a central proto-oncogenic function of ets-factors, leading to accelerated glioma growth.
Intracellular iron content, ROS production and proliferation was measured in U373 (WT) cells, tetracycline inducible etsDN expressing U373 cells (etsDN), TfR overexpressing etsDN U373 (etsDN+TfR) cells and a low malignancy astrocytoma with and without forced TfR expression (1321N1- TfR respectively WT). (A) High intracellular iron levels are observed in U373 cells having intact ets signalling or in U373 etsDN cells overexpressing TfR, but not in U373 cells expressing etsDN alone. Higher intracellular iron accumulation was also measured in 1321N1 cells overexpressing TfR compared to WT 1321N1. (B) Similarly, high oxidant levels were measured in U373 cells with intact ets signalling or in TfR overexpressing etsDN cells, but not in U373 cells expressing etsDN alone; additionally high oxidant generation was observed in TfR overexpressing 1321 cells. (C) Flow cytometry revealed increased oxidant levels in U373 cells with high TfR expression were homogenous throughout the cell populations. (D) BrdU incorporation assay for showed high proliferation levels in U373 cell having intact ets signalling or in U373 etsDN cells overexpressing TfR, but not in U373 cells expressing etsDN alone; proliferation was also drastically increased after TfR overexpression in 1321N1 cells. (E) Levels of BrdU incorporation in U373 WT cells are strongly decreased after iron (DFO) or oxidant (NAC) chelation.

6.9. The Transferrin receptor rescues clonal growth in etsDN glioma

The role of oxidant production and ets factors for cancer cell proliferation is also highlighted by another observation: U373 cells constitutively expressing etsDN do not grow at clonal level, i.e. clonal etsDN cells could only be generated by a

conditional expression system. However, when we overeexpressed TfR in bulk etsDN cultures we grow these cells at clonal density (not shown).

Overall, we showed that downregulation of TfR expression by attenuating ets activity decreased intracellular iron accumulation and redox levels in glioma. The generation of reactive oxygen species is catalyzed by excess intracellular iron and contributes to brain pathology (Zecca *et al*., 2004) and high oxidant levels can promote cancer cell proliferation (Hussain *et al*., 2003). Importantly, when we rescued TfR expression in etsDN cells we found that these glioma cells re-established not only iron and oxidants but also cell proliferation comparable to the levels in wild type cells. These data show for the first time, that modulation of redox signalling is an important pro-tumorigenic effect that can be initiated by ets transcription factors.

6.10.LMW-PTP is a target of ets/TfR mediated oxidation

After establishing that high proliferation levels in gliomas are maintained by ets-driven TfR overexpression and subsequent iron accumulation and ROS generation we investigated which molecules downstream of ROS mediate signals for the accelerated cell division rate. Hence, we searched for proteins, which are known to transduce growth signals and which are sensitive to ROS. Therefore, we electrophoretically separated whole protein contents of U373-WT, U373-estDN and U373-etsDN+TfR and in parallel performed an immunoblotting procedure for the detection of oxidised proteins (Oxyblot). From the blots we identified bands containing high amounts of differentially oxidised proteins among our cell lines. The corresponding bands were excised from gels separating the total protein contents of glioma cells and were analysed by mass spectrometry. With this procedure We identified low molecular weight protein tyrosine phosphatase (LMW-PTP; Chiarugi *et al*., 2004; Ostman *et al*., 2006) as a potential target for ROS mediated growth signalling (Fig. 6.10.A& B).

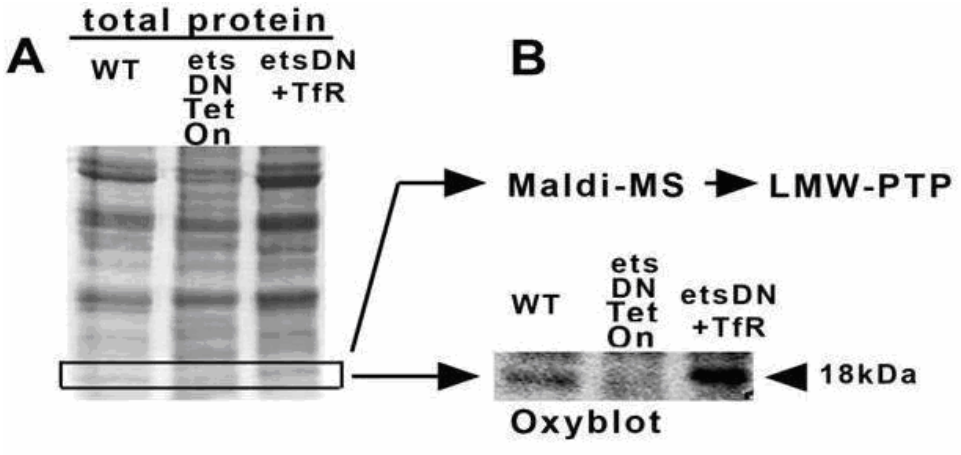
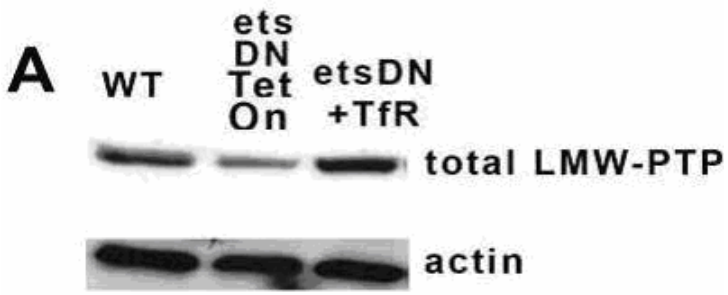


Fig. 6.10. Identification of ROS target proteins by mass spectrometry.
(A) Protein contents of WT etsDN-TetOn and etsDN+TfR cells were electrophoretically separated (total protein). (B) In parallel we performed an immunoblotting procedure for the detection of oxidised proteins (Oxyblot). From the blots we identified bands containing high amounts of differentially oxidised proteins among our cell lines. The corresponding bands were excised from gels separating the total protein contents of glioma cells and were analysed by mass spectrometry. One of the proteins in this band was identified by Maldi-MS as low molecular weight protein thyrosine phophatase (LMW-PTP).

The total amount of LMW-PTP expression was increased in U373-WT and U373-etsDN+TfR cells compared to U373-estDN (Fig. 6.10.1, A). However, LMW-PTP was also differentially oxidised (and hence inactivated) in these three lines. In cells with high TfR and ROS content (U373-WT and U373-etsDN+TfR) we detected much higher amounts of oxidised LMW-PTP than in U373-estDN (containing low TfR and ROS levels; Fig. 6.10.1, B).



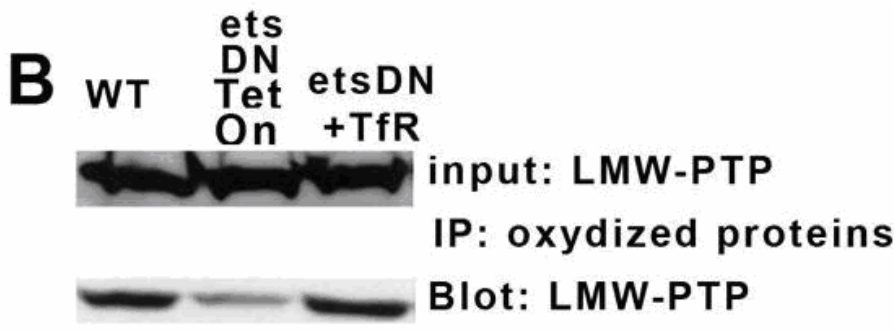


Fig. 6.10.1 LMW-PTP as a target of Ets/TfR mediated oxidation
(A) Total expression of LMW-PTP is higher in WT and etsDN+TfR cells compared to etsDN-TetOn cells. (B) Immunoprecipitation of equalized amounts of LMW-PTP (input) with the oxyblot system (IP: oxidised proteins) and immunoblot for LMW-PTP (blot: LMW-PTP), note that the amount of oxidised LMW-PTP is much higher in WT and etsDn+TfR cells as compared etsDN-TetOn.

Importantly, we observed that oxidation of LMW-PTP can account for the increased proliferation in cells with high ROS content (Fig. 6.10.2). Overexpression of a dominant negative form of LMW-PTP in which the catalytic site of the enzyme is inactivated and which therefore mimics the oxidised form of LMW-PTP (Giannoni *et al*., 2006) rescues proliferation in etsDN-TetOn cells. This suggests, that the high redox levels in glioma may facilitate growth factor signalling in the brain tumors, partly through oxidising LMW-PTP.

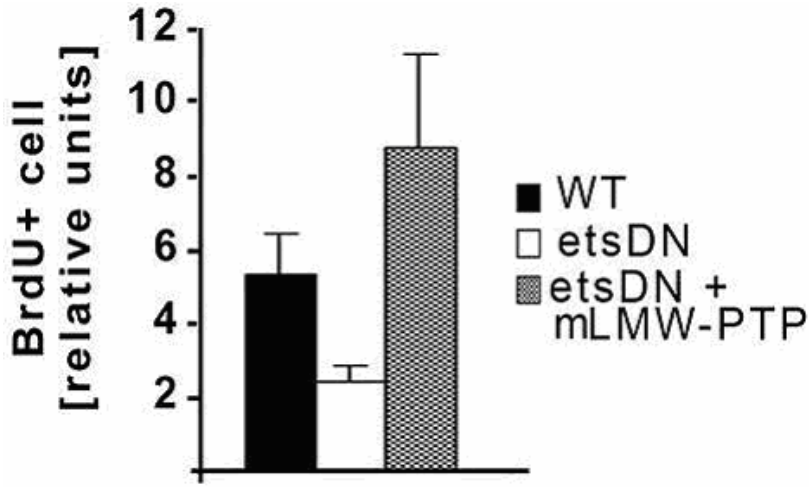


Fig. 6.10.2 BrdU proliferation assay: BrdU incorporation assay showing the proliferation of U373 EtsDN cells compared to U373 etsDN cells overexpressing a mutated (catalytically inactive) LMW-PTP and compared to (WT) U373 cells; note that the mutant LMW-PTP rescues proliferation in U373 etsDn cells

6.11. Pro-proliferative signalling via Akt is restricted to glioma cells with high ROS content

Next, we sought to establish if U373-WT and U373-etsDN+TfR have altered MAPK and Akt signalling compared to U373-etsDN cells. Indeed, cells with high TfR levels like U373-WT, U373-etsDN+TfR and U373-etsDN cells without tetracyclin exhibited high levels of phosphorylated Akt indicating the activation of the Akt pathway. Conversely, cells with low iron and ROS levels like tetracyclin stimulated U373-etsDN and DFO or NAC treated U373-WT cells had lower levels of phosphorylated Akt (Fig. 6.11.A). However, the total Akt levels in U373-etsDN were even increased compared to all other cells, suggesting some compensatory mechanism in these glioma cells, e.g. to cope with low levels of Akt phosphorylation under low ROS conditions. Akt signalling in U373 cells was Pi3Kinase dependent as indicated by the inhibition of phosphorylated Akt levels by wortmannin (Fig. 6.11. B).

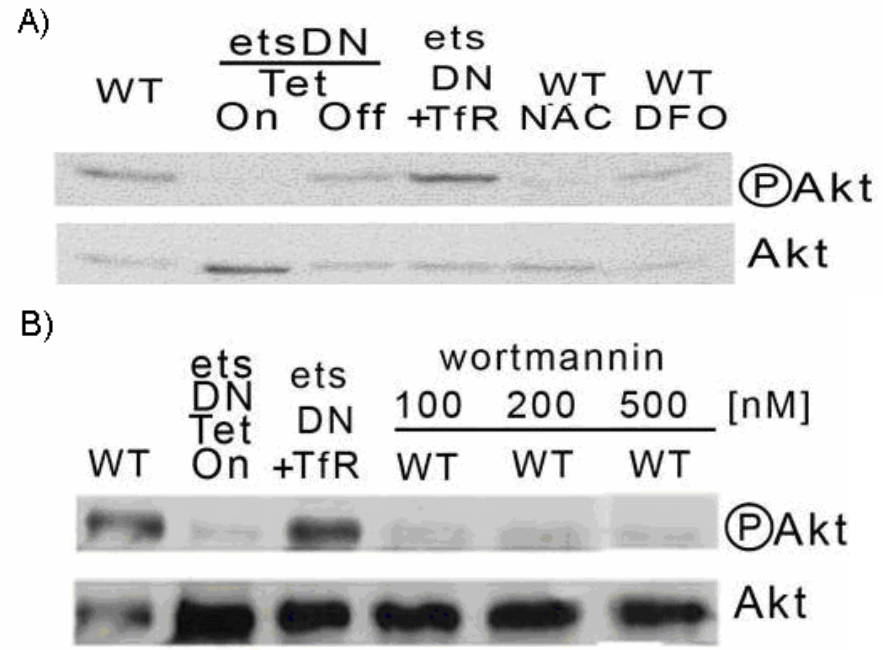


Fig. 6.11. Pro-proliferative signalling via AKT is restricted to glioma cells with high ROS content

We compared the activity of the Akt signalling by Western blotting in U373 (WT), U373 etsDn (etsDN) and etsDN+TfR U373 (etsDN+TfR) cells and investigated the effect of altered redox signalling.

(A) Levels of active Akt (phopho-AKT; P-Akt) compared to total AKT expression (Akt) in the various U373 cell lines; note that P-Akt is reduced after tetracyclin induced etsDN expression and after iron (DFO) and ROS (NAC) chelation, but remains high in all other cells. (B) P-AKT levels are reduced after application of the PI3 kinase blocker wortmannin.

6.12.Pro-proliferative signalling via MAPK is also restricted to glioma cells with TfR overexpressing glioma cells

Similar to the findings obtained for Akt phosphorylation we observed that all three major MAPK pathways are phosphorylated in cells with high iron and ROS content (WT, etsDN+TfR) and that tetracyclin induced expression of etsDN and subsequent attenuation of ROS generation abrogated MAPK signalling (Fig. 6.12). Importantly, TfR overexpressing cells showed increased activation (phosphorylation) of p38, ERK and JNK MAPKs, but the expression of total (non-phosphorylated) MAPKs was at equal level in all three cell lines or even increased in the etsDN expressing glioma. This indicates that etsDN only interferes with MAPK signalling but does not attenuate MAPK expression.

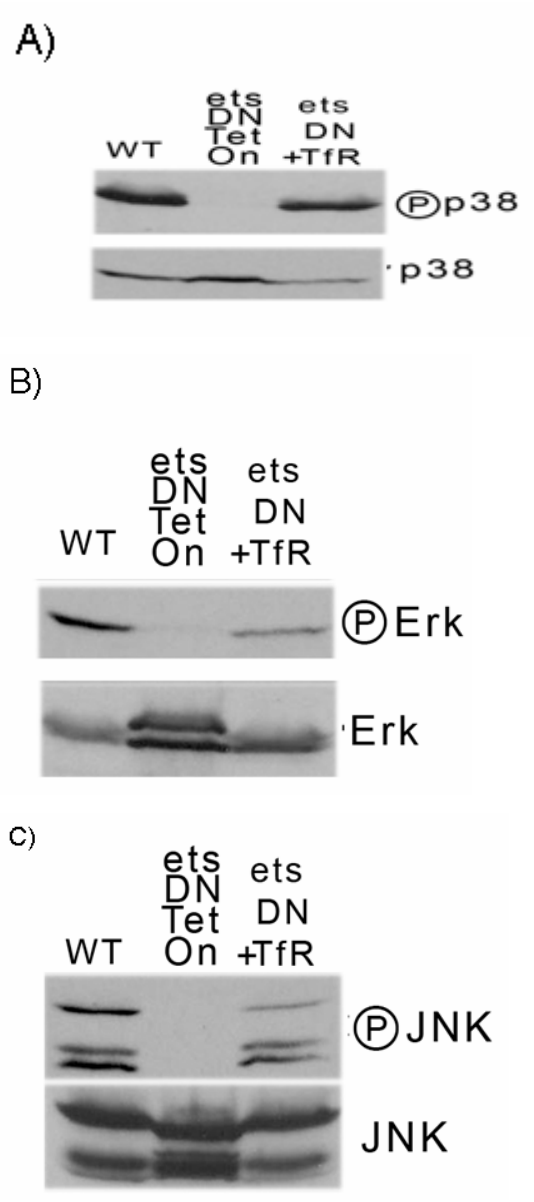


Fig. 6.12. Pro-proliferative signalling via MAPK is restricted to glioma cells with TfR overexpressing glioma cells.
We compared the activity of the MAPK signalling by Western blotting in U373 (WT), U373 etsDn (etsDN) and etsDN+TfR U373 (etsDN+TfR) cells and investigated the effect of altered redox signalling.

MAPK (A.p38, B.Erk and C.JNK) are inactive (non-phosphorylated; P-p38, p-Erk, P-JNK) after tetracyclin induced etsDN expression in U373 cells, but expression of total MAPK is not affected; note that MAPK signalling is rescued in etsDN cells after TfR overexpression (etsDN+TfR).

6.13.Effect of altered redox signalling on G_1 checkpoint control and cell cycle progression

Altered Akt and MAPK signalling can impact on cell proliferation through phosphorylation of pRB (Nath *et al*., 2003) and through phosphorylation and translocation of $p21^{(cdkn1a/waf1/cip1)}$ from the nucleus to the cytosol, which inactivates p21 (Zhou *et al*., 2001). In TfR overexpressing glioma pRB was over-phosphorylated and therefore inactivated (Fig. 6.13. A). Likewise, p21 had a predominantly cytosolic localisation in glioma cells with high TfR expression, active iron accumulation and abundant ROS generation (Fig. 6.13. B and C& D). By quantification of p21 localisation in the respective cell lines we observed that 95% of all WT cells exhibited cytosolic p21, only 19% of all tetracyclin treated etsDN-TetOn had cytosolic p21 localisation (consequently 81% of p21 was nuclear) whereas etsDN+TfR had again cytosolic p21 in 89% of all cells.
The tumor suppressors p21 and pRB control cell cycle entry in G1 and prevent cells from entering S-phase. To investigate if p21 and pRB activation in the TfR overexpressing gliomas can account for the increased proliferation of these cell lines we quantified cell cycle phases in our glioma cell lines by FACS analysis, measuring ploidity of the cellular DNA with propidium iodide. We observed that in U373-WT and U373-etsDN+TfR the amount of cells, which had entered S-phase was approximately 50% higher than in tetracyclin stimulated U373-etsDN glioma cells. These data indicate that TfR overexpression can facilitate S-phase entry and proliferation of gliomas (Fig. 6.13. E).

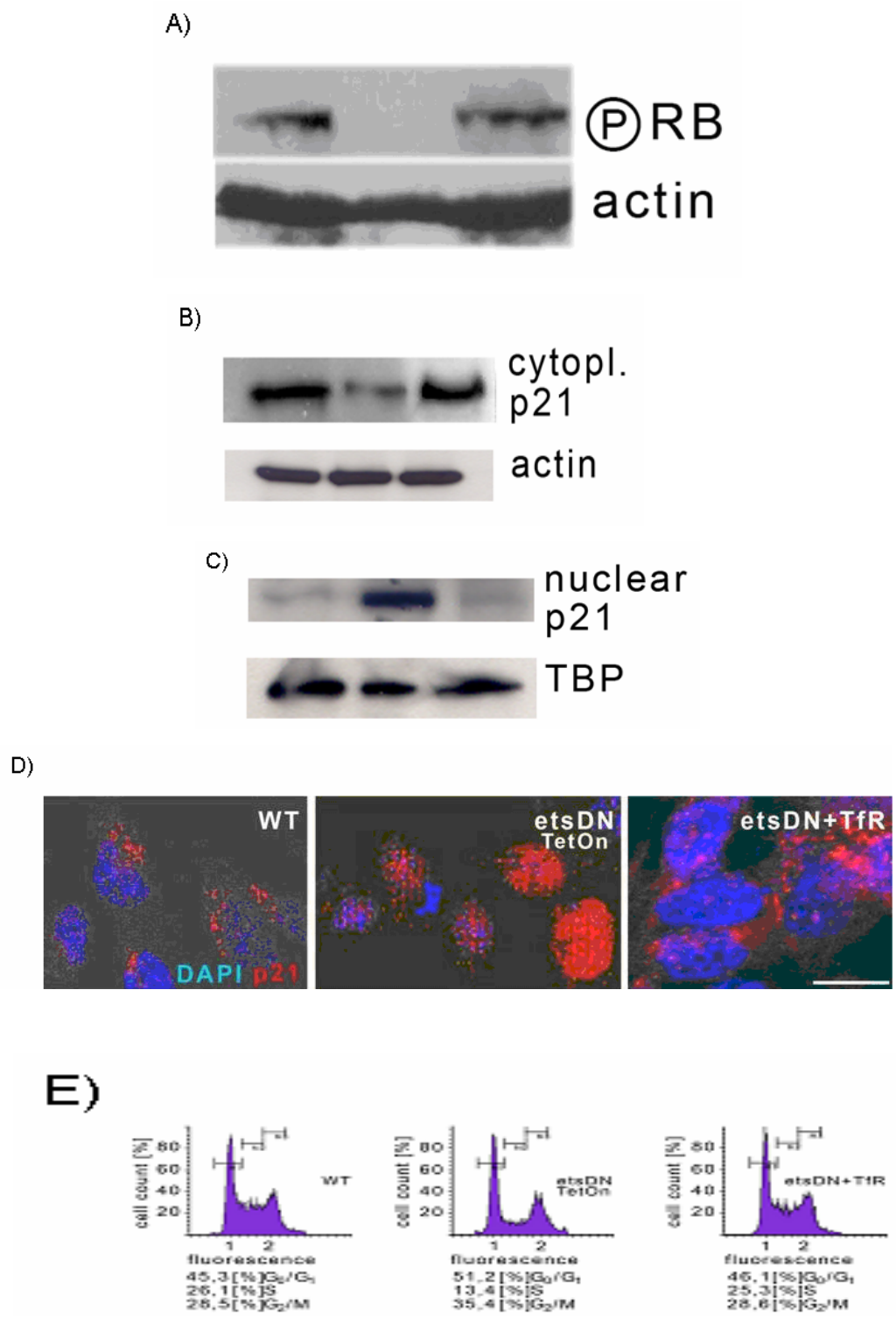


Fig.6.13. Effect of altered redox signalling on G_1 checkpoint control and cell cycle progression.

A) Phosphorylation of the RB protein (P-RB) is absent after tetracyclin induced etsDN expression, whereas pRB is strongly phosphorylated (inactivated) in U373-WT and U373-etsDN+TfR cells. Likewise, (C) p21 is active (nuclear localisation) after tetracyclin induced etsDN expression, but (B) inactive (cytoplasmic localisation) in U373-WT and U373-etsDN+TfR cells (loading controls are for cytoplasm: actin; for nuclear fraction: TATA-binding protein; TBP). (D) Immunostaining

also identifies nuclear localisation (nuclei are blue) of p21 (red) in U373-WT and U373-etsDN+TfR cells, but cytoplasmic p21 in tetracyclin induced etsDN cells.

(E) Cell cycle analysis for WT, etsDN and etsDN+TfR U373 cells by flowcytometry using propidium iodide staining; note that in WT and etsDN+TfR the number of cells entering S-phase is twice as high as after tetracyclin-induced etsDN expression.

6.14. TfR mediated, pro-proliferative redox signalling accelerates glioma growth *in vivo*

To examine if the TfR mediated increase in iron accumulation, redox signalling and cell cycle acceleration had direct consequences for the progression of glioma in vivo we subcutaneously implanted the WT cells, U373 cells overexpressing etsDN and the etsDN+TfR expressing glioma line in immune-compromised mice and monitored tumor growth over 8 weeks. Additionally, I examined if the application of DFO or NAC in the drinking water had a therapeutic effect against glioma cell growth. The tumor sizes were determined weekly. We observed that glioma with high TfR activity had the strongest growth over the whole time-course examined, whereas iron chelation by DFO greatly reduced tumor growth. In U373 cells overexpressing etsDN we detected only little tumor growth during the 8 weeks of the experiment. Most striking therapeutic results we obtained with the ROS chelator NAC, which reduced in vivo glioma growth almost to the level of etsDN expressing cells (Fig. 6.14. A).

Next, I compared the tumor sizes we had obtained by volumetric measurement over time with the actual tumor mass at the end point of our experiment. Animals were sacrificed 8 weeks after glioma cell injection and the tumor was isolated. Again the TfR overexpressing cells and glioma with active iron accumulation and redox signalling (U373-WT and U373-etsDN+TfR) had significantly increased tumor weights compared to U373 expressing etsDN and DFO or NAC treated glioma (Fig. 6.14. B). The reduced tumor growth after systemic abrogation of high iron and ROS levels by applying an iron chelator or an anti-oxidant to glioma bearing mice, further indicated the relevance of redox signalling for glioma progression.

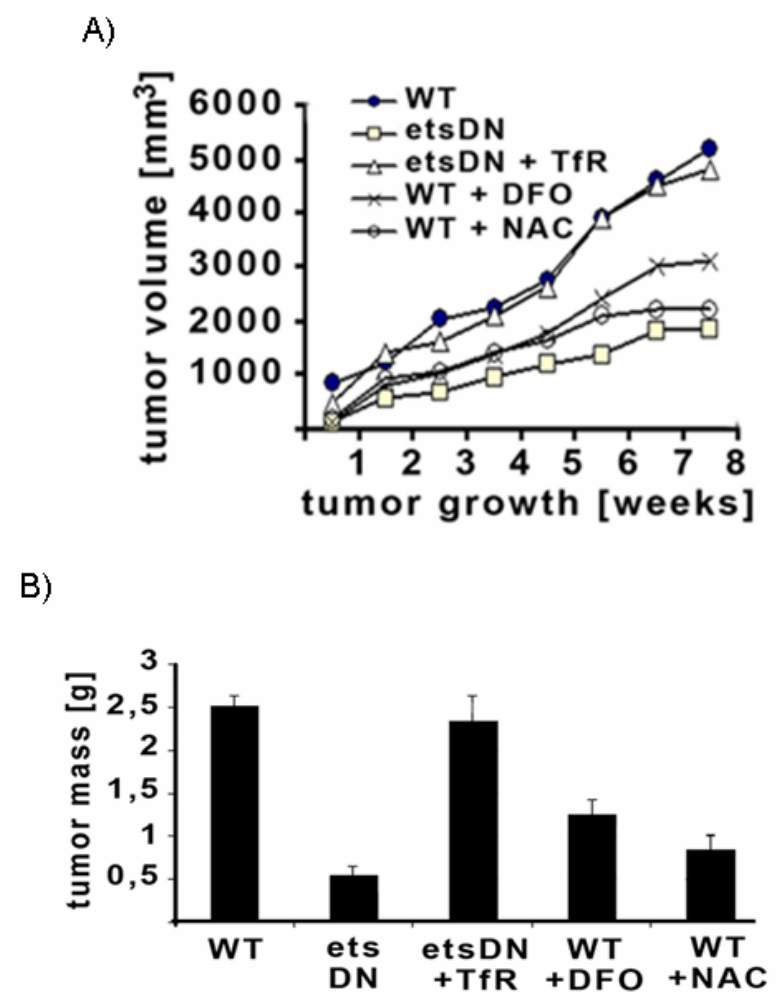


Fig. 6.14.TfR mediated redox signalling accelerates glioma growth in vivo.

(A) Tumor growth of subcutaneously implanted U373 WT, etsDn and TfR+etsDn glioma in nude mice was followed over a time course of 8 weeks. Additionally, DFO and NAC were given with the drinking water to nude mice implanted with WT cells. Glioma grew rapidly in WT and etsDN+TfR implanted mice, but progressed much slower in etsDN inoculated animals or in mice receiving DFO or NAC. (B) Finally, the tumors were resected from the mice and glioma masses were measured by weighing the resections; note that tumor masses are much higher in WT and etsDN+TfR implanted mice, but significantly lower ($p<0.001$) in etsDN inoculated animals or in mice receiving DFO or NAC with the drinking water.

6.15.Redox signalling upregulates the expression of Matrix metalloproteases

After establishing that high proliferation levels in gliomas are maintained by ets-driven TfR overexpression and subsequent redox signalling. I investigated if ets-induced TfR expression may also activate mechanism that can facilitate the expression of the growing tumor mass. Especially, I investigated if ets induced TfR may promote glioma invasion and neuronal excitotoxicity. Since gliomas grow in a

confined area (growth is ultimately limited by the skull) the tumor actively kills surrounding neurons. Additionally, single glioma cells can escape from these necrotic areas by deeply invading into the parenchyma.

To explore if ets and TfR may have a central role for glioma pathway by promoting and co-ordinating tumor growth and expansion of the tumor mass, I investigated metalloprotease expression (driving invasion) and glutamate secretion (causing neurotoxicity) in my glioma model.

Therefore, conditioned media from cultures of U373-WT, U373-etsDn and U373-EtsDn+TfR were analysed for the activity of gelatinises by gelatin zymography and a prominent band was observed in the range of approximately 65kDa in transferrin over expressing cells compared to faint bands in case EtsDn cells indicating the stronger activity of MMP-2 correlating with TfR over expression along with increased levels of free iron and ROS (Fig 6.15.).

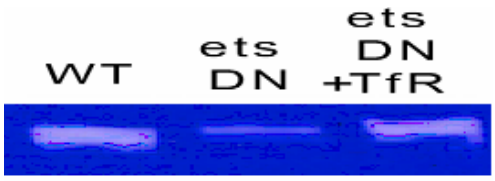


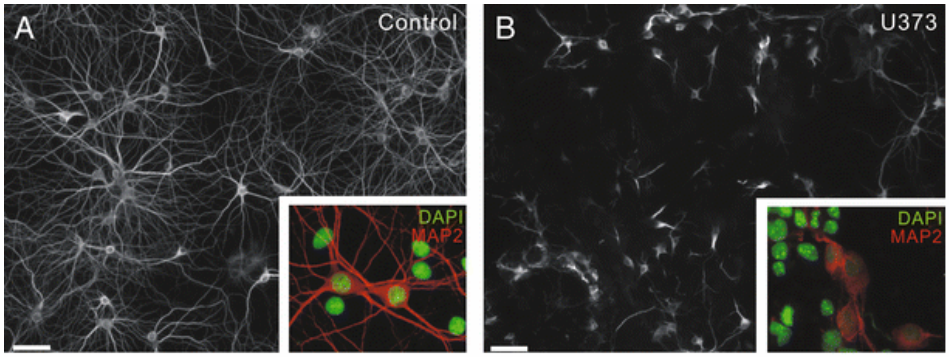
Fig.6.15. Redox signalling upregulates the expression of Matrix metalloproteases

Gelatin zymography showing the MMP2 activity in WT U373 glioma, dominant-negative U373 (etsDN) and etsDN+TfR, note that MMP2 activity is much higher in WT and etsDN+TfR.

6.16.TfR expression in glioma induces glutamate secretion and subsequently excitotoxic neuronal death

We had initially found that TfR expression is independent of iron regulatory proteins (IRPs). However, IRPs have a dual role and acquire aconitase activity when iron concentration is high (Hentze *et al*, 1996). Under high iron conditions this enzyme has a key role in the biosynthesis of glutamate (McGahn *et al*, 2005). Hence, we investigated whether the expression level of TfR is linked to glutamate release activity. Indeed, we found glioma cells with a high expression level of TfR, namely U373-WT and U373-etsDN+TfR released large amounts of glutamate into the supernatant. Glutamate levels reached 400 to 500µM within 8h a plateau value of approximately 700µM within 72h. In contrast, glioma cells with low level of TfR expression, the U373-etsDN, released only 15µM glutamate within 8h and approximately 50µM within 72h (fig 6.16.D).

To study the pathological impact of the glioma derived glutamate, we co-cultivated the tumors with hippocampal neurons. In the presence of U373-WT and U373-etsDN+TfR, we observed massive neuronal degeneration, i.e. the majority of hippocampal neurons (more than 80%) had drastically shortened or even fragmented dendrites. This effect was almost fully reversed (to about 15% of neurons with morphological changes) in cultures treated with an NMDA receptor blocker (1µM MK801) or in glioma with little TfR signalling and low iron content (U373-etsDN). Likewise, the total number of neurons was reduced in co-cultures with U373-WT and U373-etsDN+TfR gliomas (by about 45%), compared to U373-etsDN, U373+MK801 and neurons alone (fig 6.16.C)



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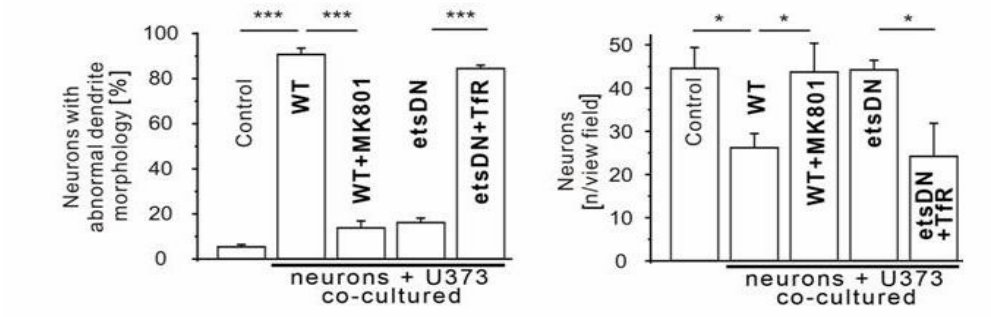

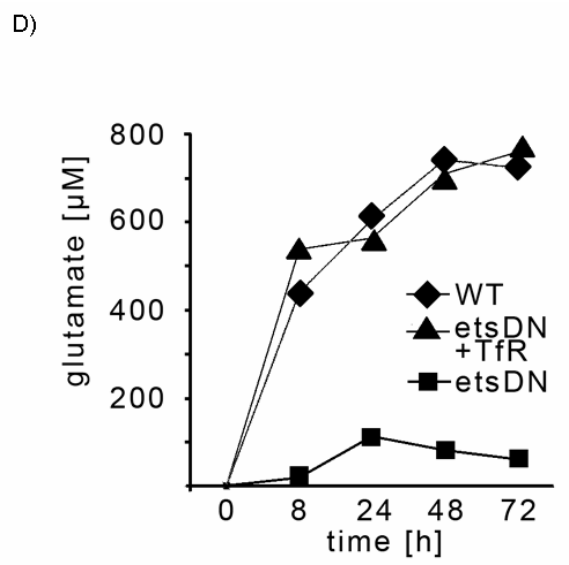

Fig.6.16. TfR overexpressing U373 gliomas induce excitotoxic neuronal death.
(A, B) MAP2 staining of hippocampal neurons cultured for 24 hours under control conditions or co-cultivated with U373 cells. (A) Control neurons without U373 cells have numerous and elongated dendrites without dendrite fragmentation. (B) In the presence of U373 cells, hippocampal neurons display a strongly altered dendrite morphology. The majority of neurons have short (if any) dendrites, often characterized by dendrite fragmentation. Astersisks denote neurons that were not classified as degenerating. (C) Quantification of the fraction of neurons with abnormal dendrite morphology cultured under different experimental conditions; note that neurons degenerate only in co-culture with TfR overexpressing glioma and that their degeneration is prevented by the glutamate-receptor (NMDA-receptor) antagonist MK801. Likewise, the total number of MAP2-positive neurons declines specifically in co-culture with TfR overexpressing glioma, this neuronal loss is prevented by MK801. (D) Time-course experiment quantifying glutamate release from U373 glioma, note that excessive glutamate release is restricted to TfR overexpressing glioma.

Significance levels (Student's t-test and ANOVA) are indicated as $P<0.05$ (*) and $P<0.001$ (***). Scale bar: 100 µm.

7. Discussion

I. Mechanism of the anti-tumorigenic effect of endogenous neural precursor cells against glioma

In the first part of my thesis I elucidated a molecular mechanism of the anti-tumorigenic effect of endogenous neural precursor cells against glioma. Recently, our group has discovered that endogenous precursor cells are attracted to experimental brain tumors and mediate an anti-tumorigenic effect by inducing glioma cell death (Glass, R. *et al*, 2005 and Walzlein, J.H. *et al* 2008). Here I provided evidence that the endogenous neural precursor cells show an additional anti-tumorigenic effect by suppressing glioma initiating cells (glioma stem cells, GSCs) via release of soluble factors like BMP7.

Neural stem and precursor cells in the adult brain are restricted to stem cell niches like the subventricular zone (SVZ) and hippocampus (Alvarez-Buylla, A., *et al* 2004). In addition to their role in brain plasticity, NPCs contribute to brain repair after injury (Hallbergson, A.F., *et al*, 2003). However, genetic mutations can transform NPCs into extremely aggressive primary brain tumors, such as glioma (Holland, E.C., *et al* 2000 and 2001). Glioma patients have an average survival time of about one year after diagnosis. Current treatment strategies are designed to target the bulk tumor mass and potentially fail to suppress an aggressive sub-population in gliomas, the so called glioma stem cells (Singh, S.K., *et al* 2004). Aggressive and recurrent glioblastomas have been shown to contain a fraction of multipotent cells with extensive self-renewing properties that are likely to drive tumor growth and a recent discovery of neural stem cell-like tumor cells, termed glioma stem cells, in GBM has created a paradigm shift in brain tumor research these cells arise from a single, self-renewing cell type, which then gives rise to the rest of the tumor, including a variety of more differentiated cell types (Singh *et al*., 2003)

These glioma stem cells can be identified, since they express the cell surface glycoprotein CD133. It is now under intensive debate that a successful glioma therapy may have to target these CD133-positive glioma stem cells. Recently it was disovered that recombinant BMP4 can suppress gliomas by specifically targeting glioma stem cells (Piccirillo, S.G., *et al* 2006), similar anti-tumorigenic effects against glioma stem cells have been reported using BMPs by an another

independent study (Lee., J *et al* 2008). During development and in postnatal neurogenesis BMPs induce NPCs to differentiate into mature glial cells (Alvarez-Buylla., *et al* 2002). This pro-differentiative role of BMPs in neural stem cells prompted us to study their roles in putative glioma stem cells.

7.1. Neural precursor cells express and release BMP7 to inhibit tumorigenic potential of glioma stem cells

To investigate further in this direction I asked by which way endogenous NPCs show their anti-tumorigenic activity towards gliomas and I have identified a NPC released, soluble factor which drastically reduces the amount of CD133 positive cells in glioma cultures of murine (GL261) and human origin (primary cultured patient material). I observed that NPC conditioned medium reduced CD133 positive glioma cells by about 50%. This effect was seen in bulk glioma cultures and in CD133 enriched cultures (containing approximately 90% glioma stem cells). Suppression of the CD133 positive cells seems to occur via induction of glioma stem cells to differentiate and also through significant induction of cell death. Further, *in vitro* the glioma stem cell suppressing factor BMP7 was identified by Western blotting, real-time PCR and ELISA. *In vivo*, I investigated BMP7 expression by immunohistochemistry and detected BMP7 in the tumor-margin, where it was located in PSA-NCAM-positive NPCs. Furthermore, I showed in cell culture that BMP7 is much more abundant in PSA-NCAM positive NPCS than in PSA-NCAM negative NPCs, that NPCs express much more BMP7 than bulk glioma or glioma stem cells and NPCs constitutively release BMP7. FACS analysis indicated that recombinant BMP7 and NPC-conditioned medium significantly reduced the number of murine and human GSCs. This effect was seen in bulk glioma cultures and in GSC-enriched cultures. Compound-C (CC, also known as dorsomorphin - a specific BMP-receptor antagonist) fully blocked the effect of recombinant BMP7 and NPC-conditioned medium. Importantly, I found that BMP7 release from NPCs suppresses the stem cell functions of GSCs: NPC-conditioned medium and rBMP7 suppressed GSCs in a sphere formation assay. Glioma stem cells generally show their strong ability to initiate tumor by their self-renewal and proliferation properties. Down regulation of undifferentiated markers like nestin and CD133 and up-regulation of differentiation markers like GFAP indicates the loss of glioma stem cell self renewal capacity. Importantly, NPC-derived BMP reduced the tumorigenic potential of glioma stem cells, which is

basically defined by their ability to form tumors from a very small number of cells and by unlimited self renewal. Firstly, BMP in NPC-conditioned medium suppressed sphere formation and expansion of GSCs in a sphere formation assay. Secondly, recombinant BMP7 and BMP from NPC conditioned medium suppressed the ability of glioma stem cells to initiate tumors from 100 intracerebrally inoculated cells.

7.2. Glioma Stem Cells respond to NPC-derived BMP7

Further I showed that glioma stem cells expresses BMP receptors for both type I and II and showed that they strongly respond to NPC-derived BMP7 as detected in the form of phosphorylated smad1/5/8 signalling and also analysed the transcriptional changes in the expression of targeted genes like Id1 and Id2 which are strongly upregulated upon treatment with NPC conditioned medium. Pre-treatment of GSCs with rBMP7 prior to brain-injection into mice strongly enhanced survival, as compared to controls receiving untreated GSCs.

7.3. NPC derived BMP7 elicits a pro-differentiation effect on glioma stem cells

BMP7 or NPC-derived BMP7 induced morphological changes in glioma stem cells like a more differentiated morphology. I also observed an increase in the expression of astroglial markers (Glial fibrillary acidic protein (GFAP) immunoreactivity), together with a reduced stemness marker nestin which suggests that BMP7 from NPC produces a pro-differentiation action on glioma stem cells (which, predominantly, acquire an astroglial-like fate) and depletes the pool of tumorigenic glioma stem cells.

Overall, my findings suggest that NPCs are attracted to glioma and that BMP7 release from NPCs suppresses CD133+ glioma stem cells. Therefore, NPC-derived BMP7 might be a promising new strategy against malignant glioblastomas inducing differentiation rather than killing the tumor-initiating glioma stem cells.

II. Cell autonomous mechanism of ets transcription factor induced pro-tumorigenic signalling in glioma

The pro-tumorigenic effects of ets transcription factors are long known (Dittmer and Nordheim, 1998) and especially the invasion promoting role of the founding member of the ets family ets1 has been described in glioma (Kita *et al*., 2001; Kitange *et al*., 1999a; Kitange *et al*., 1999b). However, it remained largely unexplored how ets factors promote glioma proliferation (Kitange *et al*., 1999a). In my present study I showed that ets factors can have profound effects on brain tumor progression by altering the redox levels in glioma via increased expression of TfR.

Increased oxidant production in gliomas occured downstream of TfR overexpression and excess iron accumulation. Increased oxidant (ROS) production in gliomas expressing TfR at high level inactivates the tumor suppressor LMW-PTP, activates the MAPK and Akt pathways, inactivates cell cycle control at the G1 checkpoint and drives glioma proliferation in vitro and in vivo. Concomitantly, TfR mediated high glutamate release, making iron accumulating glioma cells excitotoxic for neurons. Overall, the data indicate that in glioma TfR co-ordinately promotes tumor growth and the provision of space for the advancing tumor.

7.4. Transferrin receptor expression in glioma is regulated by an ets transcription factor

I detected high levels of TfR expression in virtually all proliferating cells in my glioma model and observed that proliferation indeed largely depended on TfR expression in the human glioma cells (U373 cells). Under physiological conditions the expression of TfR is regulated in a complex fashion on the transcriptional (Sieweke, M. H. *et al*, 1996, Marziali, G. *et al*, 2002) and post-transcriptional (Owen, D. *et al*, 1987) level. However, in the U373 glioma cells, TfR expression remained largely unchanged after iron chelation or iron administration. This suggests that the diverse cellular iron regulatory mechanisms, which co-control TfR mRNA stability according to the intracellular iron level under physiological conditions (Zecca, L. *et al*, 2004), have a minor role for TfR expression in glioma. Using a reporter gene assay I screened for factors exerting transcriptional control over TfR expression and observed that mainly ets factors are responsible for the elevated TfR levels in glioma. Furthermore, interfering with ets signalling strongly

attenuated TfR expression, which could be rescued to control levels by overexpressing TfR from a vector. Hif1 and hypoxia did not alter TfR expression in glioma.

7.5. Transferrin receptors control redox signaling and proliferation

Downregulation of TfR expression by attenuating ets activity decreased intracellular iron accumulation and decreased the redox level in glioma. The generation of reactive oxygen species is catalyzed by excess intracellular iron and contributes to brain pathology (Zecca, L. *et al*, 2004) and high oxidant levels can promote cancer cell proliferation (Hussain, S. P. *et al*, 2003). Proliferation of U373-etsDN cells was largely diminished compared to wild type cells, but could be fully rescued by forced TfR expression. Furthermore, the overexpression of TfR was sufficient to rescue clonal growth of etsDN cells. These data show for the first time, that modulation of redox signalling is an important pro-tumorigenic effect of ets transcription factors.

7.6. LMW-PTP is a target of TfR-mediated redox signaling

Next, I asked which molecule(s) and signalling pathways may be specifically altered by elevated oxidant levels in glioma and identified LMW-PTP as a target of ROS in brain tumor cells. I showed that oxidised LMW-PTP was much more abundant in cells expressing TfR at high level than in U373etsDN cells containing TfR only at low level. Forced expression of a point mutated (inactive) form of LMW-PTP rescued U373etsDN cell growth, showing the importance of the oxidisation (inactivation) of this molecule for glioma proliferation.

LMW-PTP is an important negative modulator of receptor tyrosine kinases like the PDGF-receptor (Tonks, 2006) and counteracts activation of the Ras/MAPK and PI3Kinase/Akt signalling pathways downstream of growth factor signalling (Giannoni *et al*., 2006; Pandey *et al*., 2007; Xing *et al*., 2007). In this part of thesis I provided evidence that the MAPK and Akt pathways are only fully active in gliomas with high TfR expression. In U373etsDN cells the low TfR expression induces only low iron accumulation that generates only low oxidant levels, which oxidises little LMW-PTP. Therefore, in U373etsDN cells the LMW-PTP can be active and yields low MAPK and Akt activation. Simple re-expression of TfR in U373etsDN gliomas fully reverts this phenotype. Elevated Akt and MAPK signalling is found in most glioma and may be one of the primary parthways

driving brain tumor initiation and progression (Holland, 2001). This study suggests that TfR mediated redox signalling is in addition to mutations of growth factor receptors and of PTEN - important for the activation of Akt and MAPK.

7.7. TfR expression triggers Akt and MAPK signaling and inhibits p21 and pRB

Akt and MAPKs exert growth control by inactivation of G1 checkpoint proteins p21 and pRB (Zhou, B.P. *et al*, 2001, Suyama, H. *et al*, 2004, Fernandes, D. J. *et al*, 2004). In glioma cells with high redox signalling (U373WT and U373etsDN+TfR cells) I observed hyperphosphorylated (inactivated) pRB, whereas U373etsDN cells contained hypophosporylated pRB, which actively blocks cell cycle entry in G1. Likewise, cells with high TfR Expression and activated Akt showed inactivation of the G1 cell cycle arrest protein p21. The tumor suppressor p21 is phosphorylated by Akt, which induces shuffling of p21 out of the nucleus and loss of tumor suppressor function (Zhou, B. P. *et al*, 2001). I observed high nuclear p21 in U373etsDN cells and high cytoplasmic p21 in U373WT and U373etsDN+TfR cells. On the contrary, the TfR-low cells (U373-etsDN) were arrested in G1 and in G2.

High activity of the MAPK pathway is not only important for checkpoint control, but can also signal to ets transcription factors, which are the nuclear effectors for many MAPK signals (Dittmer and Nordheim, 1998; Sharrocks, 2001). Consequently, I found that TfR overexpression in etsDN cells facilitated massive overexpression of a range of cancer-relevant ets factors. This level of ets overexpression appeared specifically in glioma also expressing the dominant negative ets construct plus TfR (i.e. U373etsDN+TfR cells), since only here a block in ets transcription needed to be overcome. Cells expressing etsDN alone (i.e. U373etsDN) had no means to overexpress ets factor. The expression of TfR was modulated by inducing changes in oxidant levels after application of H_2O_2 and NAC. In our model TfR appears to be downstream of ets factors and the expression of ets factors can also be redox dependent (Wilson *et al*., 2005). Hence, there is a possibility that ets mediated TfR expression increases oxidants that finally induce ets factors again. The increased cell cycle arrest in TfR-low cells explains the results from our BrdU assay, which showed that U373etsDN cells proliferate less than U373WT and U373etsDN+TfR cells.

7.8. TfR-mediated redox signaling accelerates glioma growth *in vivo*

To see if glioma growth is also redox dependent in vivo, I measured subcutaneous growth of our human glioma lines in immune-compromised mice. Indeed, tumors were growing at much higher pace in glioma expressing TfR at high level. Systemic abrogation of high iron and ROS levels by applying an iron chelator or an anti-oxidant yielded reduced tumor growth, further indicating the relevance of redox signalling for glioma progression.

7.9. TfR expression in glioma cells induces glutamate secretion and NMDA-receptor-mediated reduction of neuron mass

Accelerated proliferation rates in glioma could not lead to continous glioma progression without coordinated provision of space for the expanding tumor mass. On this point, glioma have requirements different from other solid tumors, since the skull prevents the brain tissue to be invariably be pushed aside. Gliomas circumvent this problem by actively creating space for their own growth, i.e. by killing surrounding neurons (Takano, T. *et al*, 2001, Sontheimer, H. 2003). I show that TfR promotes both, excessive proliferation and massive reduction of neuron mass, which most likely reflects NMDA-receptor-mediated excitotoxicity. Overall, our data help to explain the long standing observation that iron promotes neural tumor cell cycles (Jeitner and Renton, 1996; Renton and Jeitner, 1996) and growth of glioma (Basset *et al*., 1985). Furthermore, this study suggests that the increased transferrin receptor expression (Recht *et al*., 1990) and the augmented iron accumulation (Mykhaylyk *et al*., 2005) which are reported especially for high grade glioma are not mere by-products of a disturbed metabolism, but are a driving force for tumor proliferation

Hence, TfR represents an important target for glioma therapy, by which neural tumor cell cycles (Renton, F. J. *et al*, 1996, Jeitner, T. M. *et al*, 1996), glioma expansion (Basset, P. *et al*, 1985) and neurological problems - that are frequently occurring with these tumor – may be suppressed.

8. Summary

8.1. Neural precursor cell-derived BMP7 inhibit the tumorigenic potential of glioma stem cells

In the first part of my thesis I described the molecular mechanism of the anti-tumorigenic effect of endogenous neural precursor cells against glioma. As from the recent findings from our group that endogenous neural precursor cells (NPC) are attracted to experimental brain tumors (gliomas) and induce tumor cell death (Glass, R. *et al*, 2005 and Walzlein, J.H. *et al* 2008). Here, I report that NPC exert an even more far-reaching anti-tumorigenic effect: NPC migrate to murine and primary-human CD133-positive glioma stem cells (GSC) and suppress GSC via BMP7 release.

In vivo, I investigated BMP7 expression by immunohistochemistry and detected BMP7 in the tumor-margin, where it was located in PSA-NCAM-positive NPCs. Real-time PCR and ELISA of cultured cells revealed that BMP7 is most abundantly expressed in PSA-NCAM positive NPC. Constitutive BMP7 release from cultivated NPCs was detected by ELISA. FACS analysis indicated that recombinant BMP7 and NPC-conditioned medium significantly reduced the number of murine and human GSCs. This effect was seen in bulk glioma cultures and in GSC-enriched cultures. Compund-C (a specific BMP-receptor antagonist) fully blocked the effect of recombinant BMP7 and NPC-conditioned medium. Importantly, I found that BMP7 release from NPCs suppresses the stem cell functions of GSCs which suppressed proliferation and self-renewal capacity of GSCs there by reducing their tumor initiating ability.

Further I showed that glioma stem cells expresses BMP receptors for both type I and II and showed that they strongly respond to NPC-derived BMP7 as detected in the form of phosphorylated smad1/5/8 signalling and also analysed the transcriptional changes in the expression of targeted genes like Id1 and Id2 which are strongly up-regulated upon treatment with NPC conditioned medium or with rBMP7.

Pre-treatment of GSCs with NPC conditioned medium or rBMP7 prior to brain-injection into mice strongly enhanced survival, as compared to controls receiving untreated GSCs.

Overall, these findings suggest that neural precursor cells are attracted to glioma and that NPC-derived BMP7 inhibit the tumorigenic potential of glioma stem cells.

8.2. Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells

In the second part of my study I investigated cell autonomous events in glioma that are important for tumor progression and described a mechanism for ets transcriptor factor induced pro-tumorigenic signalling in glioma. Role of ets family transcription factors especially ets1- has been described in glioma (Kita *et al*., 2001; Kitange *et al*., 1999a; Kitange *et al*., 1999b) and further the protumorigenic effects of ets transcription factors are long known (Dittmer and Nordheim, 1998). However, it remained largely unexplored how ets factors promote glioma proliferation (Kitange *et al*., 1999a). In my study I showed that ets factors can have profound effects on brain tumor progression by altering the redox levels in glioma via increased expression of TfR. Transferrin receptors (TfR) are overexpressed in brain tumors, but the pathological relevance has not been fully explored. Here, I provided evidence that TfR is an important downstream effector of ets transcription factors that promotes glioma proliferation and increases glioma-evoked neuronal death. Increased oxidant production in glioma expressing TfR at high level inactivates the tumor suppressor LMW-PTP, activates the MAPK and Akt pathways, inactivates cell cycle control at the G1 checkpoint and drives glioma proliferation in vitro and in vivo. Furthermore, my data suggest that the elevated redox potential reciprocally promotes the expression of TfR, potentially creating a positive feedback mechanism in which high amounts of ROS and TfR perpetually induce each other and drive glioma proliferation (Fig.8).

Overall, my results help to explain the long standing observation that iron promotes neural tumor cell cycles (Jeitner and Renton, 1996; Renton and Jeitner, 1996) and growth of glioma (Basset *et al*., 1985). Furthermore, our study suggests that the increased transferrin receptor expression (Recht *et al*., 1990) and the augmented iron accumulation (Mykhaylyk *et al*., 2005) which are reported especially for high grade glioma are not mere by-products of a disturbed metabolism, but are a driving force for tumor proliferation. Concomitantly, TfR mediated high glutamate release making iron accumulating glioma cells excitotoxic for neurons. Overall, the whole data indicate that in glioma that TfR

promotes glioma progression by two mechanisms, an increase in proliferation rate and glutamate production, the latter mechanism providing space for the progressing tumor mass.

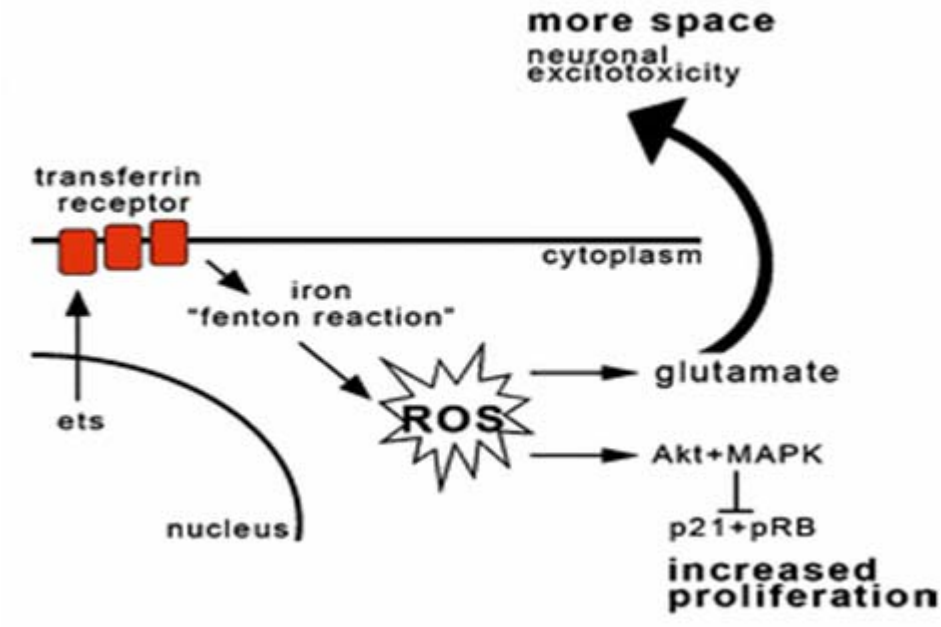

Fig.8. Schematic drawing summarising the findings from the part of Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells. Ets-factors induce TfR expression, TfR activity increases the labile iron pool and catalyses reactive oxygen formation (ROS), ROS facilitates proto-oncogenic pathways (MAPK, Akt) and blunts tumor suppressor pathways (p21, pRB) leading to accelerated growth; simultaneously ROS enables glutamate synthesis and release which leads to neuronal loss, i.e. creates space for tumor expansion (details, see text).

9. References

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10.Zusammenfassung

10.1. Neuronale Stamm- und Vorläuferzellen sezernieren BMP7 und inhibieren damit die Tumorigenität von Gliomstammzellen.

Im ersten Teil meiner Arbeit habe ich den molekularen Mechanismus beschrieben, mit dem endogene neuronale Vorläuferzellen antitumorigen gegen Gliomstammzellen wirken. Unsere Forschungsgruppe hat in bereits veröffentlichten Arbeiten gezeigt, dass neuronale Vorläuferzellen zu experimentellen Gehirntumoren migrieren und Tumorzelltod induzieren können (Glass, R *et al*., 2005; Wälzlein, J.H. *et al*. 2008). In der nun vorliegenden Arbeit zeige ich, dass die neuronalen Vorläuferzellen nicht nur benefiziell gegen die Hauptpopulation der Tumorzellen wirken, sondern darüber hinaus auch die kleinere Population der sehr aggressiven Tumorstammzellen – mittels Sekretion von BMP7 – supprimieren.

Per Immunhistochemie habe ich nachgewiesen, dass BMP7 in den tumorrandständigen neuronalen Vorläuferzellen exprimiert ist (hier vor Allem in den migrierenden PSA-NCAM-exprimierenden Vorläuferzellen). BMP7 mRNA Expression habe ich in Neurosphärenkulturen nachgewiesen und konnte zudem zeigen, dass das BMP7-Protein vor Allem in den PSA-NCAM-positiven Zellen angereichert wird. Darüber hinaus wird BMP7 von neuronalen Vorläufern in Zellkultur kontinuierlich sekretiert. Gliomstammzellen besitzen die Rezeptoren für BMPs und werden durch BMPs aus neuronalen Vorläufern zur Phosphorylierung eines BMP-spezifischen „second-messengers“ (smad1/5/8) angeregt. Die verschiedenen Gliomstammzellen, die ich in meinen Experimenten verwendete, reagieren auf BMPs aus neuronalen Vorläufern mit Zelldifferenzierung und einer Abnahme ihrer Tumorigenität. Insgesamt zeigt meine Arbeit, dass neuronale Vorläuferzellen die Pathogenität der Gliomstammzellen unterdrücken.

10.2. Transferrin-Rezeptor vermittelter Eisentransport kontrolliert die Proliferation und die Glutamat-Sekretion von Gliomzellen

Im zweiten Teil meiner Arbeit habe ich einen zellautonomen Mechanismus untersucht, der Gliomzellen in vitro und in vivo vermehrt expandieren lässt. Meine Ergebnisse zeigen, dass die Familie der ets-Transkriptionsfaktoren (und hier speziell ets1) Gliomzellen zur Proliferation anregen, indem sie die Expresion eines

Eisentransporters (dem Transferrin-Rezeptor-1; CD71) induzieren und damit die intrazelluläre Akkumulation von Eisenionen begünstigen. Eisenionen fungieren als Katalysatoren für die Bildung von Sauerstoffradikalen (Fenton-Reaktion) und verändern das zelluläre Redox-Gleichgewicht. Meine Daten beweisen, dass die Sauerstoffradikale aus der Fenton-Reaktion eine bestimmte Phosphatase inhibieren (LMW-PTP), woraufhin der MAP-Kinase Signaltransduktionsweg (und dadurch vermittelt auch der Zellzyklus) angeschaltet werden. Insgesamt proliferieren die Gliomzellen dadurch besser, zusätzlich hat dieser Mechanismus aber auch eine zweite wichtige pathogene Eigenschaft. Die Veränderung des Redox-Gleichgewichts in den Gliomzellen (via ets1 und CD71 vermittelt) regt die Tumore zu verstärkter Sekretion von Glutamat an. Dadurch werden die Gliome sehr zytotoxisch und induzieren Zelltod in den Zellen des tumorumgebenden Parenchyms (vor Allem in Neuronen). Das untergegangene Nervengewebe schafft damit den Platz, den der Tumor zur Expansion braucht. (Da der Schädel das Gehirn auf allen Seiten begrenzt, muss der Tumor sich auf diese Art Platz schaffen).

Insgesamt zeigt meine Arbeit, dass die ets1-induzierte CD71 Expression nicht nur das Tumorwachstum befördert, sondern (eng koordiniert mit dem Proliferationsprozess) auch den Platz zum Tumorwachstum schafft.

Schlagworte:

Glioma stem Zelle, BMP-7, Transferrin Rezeptor, Ets, Reactive oxygen spezies.

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List of publications

1. Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells.
Chirasani SR, Markovic DS, Synowitz M, Eichler SA, Wisniewski P, Kaminska B, Otto A, Wanker E, Schäfer M, Chiarugi P, Meier JC, Kettenmann H, Glass R.
Journal of Molecular Medicine. 2009 Feb; 87(2):153-67. Epub 2008 Dec 9.

2. Glioma induce and exploit microglial MT1-MMP expression for tumor expansion.
Markovic DS, Vinnakota K, **Chirasani S**, Synowitz M, Raguet H, Stock K, Sliwa M, Lehmann S, Kälin R, van Rooijen N, Holmbeck K, Heppner FL, Kiwit J, Matyash V, Lehnardt S, Kaminska B, Glass R, Kettenmann H.
Proc Natl Acad Sci U S A.(PNAS) 2009 Jul 28; 106(30):12530-5.

3. BMP-release from endogenous neural precursors attenuates tumorigenicity of glioma initiating cells
Chirasani, S.R., Sternjak, A., Wend, P., Momma, S., Herold-Mende, C., Besser, D., Synowitz, M., Kettenmann, H., Glass, R.
(Manuscript in review).

4. Smad7 regulates the adult neural stem/progenitor cell pool in a TGF-beta and BMP-independent manner.
Krampert, M., **Chirasani, S.R**., Wachs, F.P., Bogdahn, U., Heuchel, R., Aigner,L.
Molecular and Cellular Biology. (Manuscript under review).

5. Chaudhry, R., Nisar, N., Hora, B., **Chirasani, S.R**., Malhotra, P.
Expression and immunological characterization of the carboxy-terminal region of the P1 adhesin protein of *Mycoplasma pneumoniae.*
Journal of Clinical Microbiology, 2005; 43(1): 321-5.

International conferences attended:

1. 6th forum of European Neurosciences **(FENS)**, July 12-16, 2008 **Geneva, Switzerland.**

Poster Presentation: Glioma induce and exploit microglial MT1-MMP expression for tumor expansion.

2. Society for neuroscience conference (SFN-2007) in **San Diego (USA)** from November 2nd –8th, 2007

Poster Presentation: Transferrin receptor mediated redox signalling promotes mapkinase activity and glioma growth

2. 5th forum of European Neurosciences **(FENS)**, July 8-12, 2006 **Vienna, Austria.**

Poster Presentation: Ets-1 dependent expression of transferrin receptors in glioma mediates iron accumulation, reactive oxygen species generation and tumor progression.

3. **Brain tumor-2006**, Berlin, Germany, Poster presentation: Role of Transferrin receptors in regulating redox status in glioma progression.

4. 8th MDC/FMP PhD Student Retreat-September 2006, "Walking Molecular Pathways", Motzen, Germany

Poster presentation: Role of Ets transcription factior in regulation of transferrin receptors in glioma.

Eidesstattliche Erklärung

Ich, Sridhar Reddy Chirasani, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Cellular and molecular mechanisms of glioma growth control“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Berlin, den

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Sridhar Reddy Chirasani